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TESIS DOCTORAL

Factores bióticos y abióticos responsables de la distribución e incidencia de *Batrachochytrium dendrobatidis* en poblaciones de anfibios de zonas templadas

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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FACTORES BIÓTICOS Y ABIÓTICOS RESPONSABLES DE LA DISTRIBUCIÓN E INCIDENCIA DE *BATRACHOCHYTRIUM DENDROBATIDIS* EN POBLACIONES DE ANFIBIOS DE ZONAS TEMPLADAS

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Imagen de la portada: *Alytes obstetricans* en acuarela.
Cristina Sausor

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ACERCA DEL IDIOMA

La presente tesis doctoral ha sido redactada en dos idiomas: inglés y español. El apartado de *Introducción* ha sido redactado en español y los *Capítulos 2-9*, en inglés (idioma original en que se escribieron los artículos para su posterior publicación en revistas científicas internacionales). Se incluye un resumen al inicio de cada capítulo en español. Los apartados *Resumen/Abstract* y *Conclusiones/Conclusions* han sido redactados en los dos idiomas.

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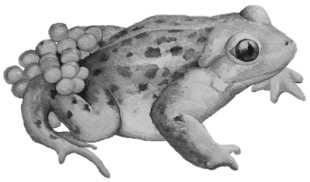
A mi padre

“El conocimiento empieza en el asombro”

SÓCRATES

INDICE DE CONTENIDOS



RESUMEN/ABSTRACT

RESUMEN

El concepto de enfermedad ha evolucionado mucho durante los últimos siglos. Actualmente, sabemos que las enfermedades están presentes de manera natural en todos los sistemas ecológicos y se consideran un factor regulador de la diversidad biológica, a través de fenómenos de competencia, cambios en la distribución de especies o en procesos evolutivos como la especiación. Las enfermedades causadas por organismos infecciosos ejercen presiones selectivas sobre el hospedador, que puede llegar a ser capaz de desarrollar adaptaciones que le permitan convivir con el patógeno en el mismo espacio-tiempo. El problema surge cuando un determinado patógeno es introducido de repente y de forma artificial en un lugar donde los hospedadores potenciales no han desarrollado una inmunidad específica para combatirlo. En este caso hablamos de enfermedades emergentes, y son una de las principales causas de la pérdida de biodiversidad acelerada que estamos viviendo en la actualidad.

Entre los vertebrados, los anfibios son uno de los grupos más amenazados, por delante de aves y mamíferos. En este caso, hongos y virus de reciente aparición están siendo responsables de su rarefacción en todo el mundo. Esta tesis se centra en el estudio del hongo patógeno *Batrachochytrium dendrobatidis* (en adelante *Bd)*, del orden Chytridiales, cuya reciente expansión está afectando gravemente a multitud de especies de anfibios. El primer declive conocido en Europa por este hongo ocurrió en la Sierra de Guadarrama en el período 1997-2002 y desde ese momento, no han cesado los esfuerzos encaminados a frenar su expansión, dilucidar su origen y entender la dinámica de la infección (una revisión del conocimiento existente en España hasta la fecha se recoge en la Introducción, Capítulo 1). La presente tesis doctoral forma parte de esos esfuerzos, y tiene como objetivos generales recabar información sobre la distribución del patógeno en algunas áreas críticas de la Península Ibérica y del Norte de Marruecos (Bloque I), así como analizar diferentes factores bióticos y abióticos que modulan la dinámica y las consecuencias de la enfermedad (Bloque II). Este segundo bloque de estudios identifica a la quitridiomicosis como una enfermedad capaz de modificar los fenómenos de competencia inter-específica, de producir cambios en la distribución de las especies, e incluso de cambiar la estructura genética de las poblaciones afectadas interfiriendo, por tanto, en sus procesos evolutivos. Los objetivos específicos de esta tesis se describen a continuación, y se abordan en cada capítulo con una metodología determinada. No obstante, en todos los casos donde ha sido necesario detectar la presencia del hongo o cuantificar la

carga de infección en muestras recogidas en campo se ha utilizado la técnica de la reacción en cadena de la polimerasa a tiempo real en laboratorio (*qPCR, quantitative Polymerase Chain Reaction*).

La especial susceptibilidad de las especies del género *Alytes*, así como la de otras especies endémicas con distribuciones restringidas y localizadas en zonas favorables al desarrollo de la quitridiomicosis, motivó el estudio de la situación en ciertas áreas geográficas (Bloque I). En el norte de Marruecos, el sapo partero marroquí (*Alytes maurus*), y en el sur peninsular, el sapo partero bético (*Alytes dickhilleni*), están restringidos casi completamente a zonas montañosas idóneas para el desarrollo de la enfermedad. Los muestreos realizados en Marruecos determinaron la presencia de *Bd*, por primera vez en el norte del continente africano, en varias especies y distintas zonas del país (Capítulo 2) y, aunque en ese momento no se constató la infección en *A. maurus*, muestreos más recientes han confirmado mortalidad por *Bd* en la especie. En el sur peninsular, los muestreos llevados a cabo en toda la distribución de *A. dickhilleni* detectaron la presencia de *Bd* en dos zonas muy alejadas entre si, y con una prevalencia del 100%, sugiriendo una introducción reciente con escasa dispersión. Aunque en ese momento no se detectó mortalidad en el campo, ésta fue superior al 70% en laboratorio, confirmándose el riesgo que supondría la expansión de *Bd* para la especie (Capítulo 3). De hecho, en los últimos años, *Bd* se está extendiendo por toda el área de distribución del sapo partero bético, y las primeras mortalidades masivas registradas han dado paso a la casi total extinción de varias poblaciones, que resultaban clave para la especie.

Paralelamente, también se llevaron a cabo muestreos en todas las poblaciones existentes de *Calotriton arnoldi*, el tritón del Montseny, catalogado como en peligro de extinción en el Catálogo Español de Especies Amenazadas, aunque en este caso, con resultados negativos en todas las muestras (Capítulo 4).

Las condiciones ambientales determinan completamente el impacto de *Bd* sobre las poblaciones de anfibios, por lo que uno de los primeros objetivos fue abordar el estudio del principal factor abiótico implicado en la dinámica de la enfermedad. Las temperaturas bajas, a una corta escala temporal, resultaron ser el mejor predictor de la intensidad de la infección en poblaciones de *A. obstetricans* infectadas en zonas de baja altitud de Zamora (Capítulo 5). Tras realizar un seguimiento exhaustivo de seis poblaciones durante un año completo y registrando, tanto la temperatura

del agua como la intensidad de la infección de las larvas, se obtuvo un marcado patrón estacional. La infección se correlacionaba inversamente con la temperatura, presentando sus valores más altos (tanto en intensidad como en prevalencia de infección), en los meses más fríos del invierno. Dado que las temperaturas registradas se encontraban fuera del rango de crecimiento óptimo del patógeno *in vitro*, estos resultados se explican explican mejor por la falta de capacidad de las larvas para hacer frente a la infección bajo condiciones ambientales extremas que por la idoneidad de estas condiciones para el desarrollo del hongo.

En el Parque Nacional de la Sierra de Guadarrama, el rápido y severo declive sufrido por la especie más afectada, el sapo partero común (*Alytes obstetricans),* condujo a la puesta en marcha de un programa de cría en cautividad. Para el establecimiento de la colonia reproductora, se capturaron algunos de los escasos ejemplares que sobrevivieron a la epidemia. Paralelamente, y para constatar la idoneidad de estos ejemplares como parentales del programa de cría y diseñar los cruzamientos adecuados, se realizó un estudio genético utilizando marcadores moleculares (microsatélites) en los núcleos reproductores remanentes (Capítulo 6). Como resultado del análisis de la estructura genética de la población de *A. obstetricans* del macizo de Peñalara, se observó una baja variabilidad genética, con claras evidencias de haber pasado por un fuerte cuello de botella como consecuencia de la enfermedad. Este hecho, unido a la relativamente baja capacidad de dispersión de los anfibios y a la presencia de barreras geográficas, se asoció con la fuerte estructura genética observada en la población. Por lo tanto, para recuperar la diversidad genética original y evitar una depresión por endogamia, se recomienda utilizar una mezcla de los ejemplares de las distintas procedencias como fuente de organismos para futuras reintroducciones. Los diferentes núcleos remanentes pueden ser considerados como una única población, exceptuando uno de ellos con el que parece no existir intercambio genético y que se corresponde con el núcleo poblacional más alejado geográficamente.

Dado que la incidencia de la quitridiomicosis varía enormemente entre especies y con objetivo de evaluar el papel que juega cada una dentro de la comunidad de anfibios en la transmisión y dinámica de la enfermedad, se realizaron infecciones experimentales en laboratorio y experimentos en campo. Los resultados corroboran el papel de *A. obstetricans* como principal reservorio de la infección y reflejan como su presencia es responsable del aumento de la carga de zoosporas en las demás especies por transmisión directa, comportándose como un *super-hospedador*.

Por otro lado, se pone de manifiesto la diferente susceptibilidad a la infección en todas las especies estudiadas (Capítulo 7).

La práctica desaparición de las grandes larvas invernantes de *A. obstetricans* de las lagunas de Peñalara motivó la expansión de *Bufo spinosus*, el sapo espinoso común, al ocupar las lagunas permanentes que habían quedado vacías. El seguimiento para verificar y cuantificar este fenómeno se realizó con el método de marcaje y recaptura en diferentes sesiones de muestreo, introduciendo un microchip identificativo a más de 1.500 individuos durante 5 años para analizar posteriormente los parámetros demográficos de la población (Capítulo 8). El resultado de este estudio confirma las sospechas de las observaciones de campo. La elevada mortalidad observada en las orillas de las lagunas en el momento de la metamorfosis, se traduce en una baja tasa de reclutamiento (inferior a la estimada para otros sapos que viven en el mismo ambiente), que no es suficiente para compensar la tasa de supervivencia de la especie, condenando a la población a un lento, pero inexorable declive.

Como se deduce del párrafo anterior, *Bd* es capaz de alterar la estructura de toda una comunidad de anfibios debido a su capacidad de infectar diferentes hospedadores y a la diferente susceptibilidad de los mismos ante la infección. La puesta en marcha de programas de seguimiento a largo plazo para registrar los cambios en las tendencias poblacionales de las diferentes especies, ha resultado ser una manera muy útil para conocer lo que ha sucedido en el Macizo de Peñalara, tras la irrupción de *Bd* (Capítulo 9). Desde 1999, año en el que se inició el programa de seguimiento, se ha realizado un intenso estudio de tendencias poblacionales mediante la realización de hasta 6 visitas, a lo largo de la estación reproductora, a los 29 sectores de puntos de agua catalogados. En dichas visitas se realizaron conteos de larvas y/o de puestas (dependiendo de la especie) en las casi 250 charcas y lagunas catalogadas, siendo asignadas a las distintas categorías de abundancia establecidas. El análisis de las tendencias poblacionales revela que sólo 3 especies han sufrido declives importantes, mientras que otras especies permanecen estables o incluso han experimentado crecimientos poblaciones, relacionados con cambios ambientales.

La presente tesis doctoral revela, por un lado, la enorme complejidad a la que nos enfrentamos en la lucha contra este patógeno y por otro, la necesidad de aproximaciones multifactoriales para abordar su estudio. La capacidad de *Bd* para expandirse rápidamente a través de diversos vectores,

la intensidad de los declives que produce, el amplio rango de hospedadores que es capaz de infectar y la diferente susceptibilidad de los mismos han resultado ser factores clave para entender la dinámica de la enfermedad. Sin olvidar, la influencia de las variables ambientales modulando estas variaciones. Por último, esta tesis sienta un precedente en el análisis de la incidencia de las enfermedades emergentes, describiendo las líneas básicas de estudio ante la probable llegada de nuevas especies de patógenos con consecuencias potencialmente nefastas para la biodiversidad.

ABSTRACT

The concept of disease has evolved greatly over the last few centuries. Currently, we know that diseases are naturally present in all ecological systems and are considered as a key factor regulating biodiversity through competition phenomena, with consequent changes in the distribution of species, and modulating evolutionary processes such speciation. Diseases caused by infectious organisms exert selective pressure on the hosts that may be able to develop adaptations allowing them to coexist with the pathogens in the same space-time. The problem arises when a pathogen is introduced, suddenly and artificially, in a place where the potential hosts have not developed a specific immunity. In that case we refer to *emerging diseases*, which are one of the main causes of accelerated biodiversity loss that we are witnessing nowadays.

Among vertebrates, amphibians are one of the most threatened animal groups today, ahead of birds and mammals. Their rarefaction around the world is in great part related to newly emerging fungi and viruses. This thesis focuses on the study of the pathogenic fungus *Batrachochytrium dendrobatidis* (hereinafter *Bd*), of the order Chytridiales, whose recent expansion is seriously affecting to multitude of amphibian species. The first decline known in Europe for this fungus occurred in the Sierra de Guadarrama in the period 1997-2002, and since then, efforts to curb its expansion, elucidate its origin and understand the dynamics of infection have not ceased (a review of the knowledge existing in Spain in that sense to date is shown in the Introduction, Chapter 1). This doctoral thesis is part of these efforts and its general objectives are to collect information on the distribution of the pathogen in some critical areas of the Iberian Peninsula and North of Morocco (Block I), as well as to analyse different biotic and abiotic factors that are modulating the dynamics and consequences of the disease (Block II). This second block of studies identifies chytridiomycosis as a disease capable of: modifying the phenomena of interspecific competition; producing changes in the distribution of species and even, changing the genetic structure of the affected populations, thus, interfering in their evolutionary processes. The specific objectives of this thesis are described below, and are addressed in each chapter with a specific methodology. Nevertheless, in all cases where it has been necessary to detect the presence of the fungus or to quantify the infection load in samples collected in the field, we used the same technique in the laboratory: *qPCR* (*quantitative Polymerase Chain Reaction*).

The particular susceptibility of species of the genus *Alytes*, as well as the existence of endemic species with restricted distributions in optimal areas to the development of chytridiomycosis, motivated the survey in strategic geographic areas (Block I). In northern Morocco, the moroccan midwife toad (*Alytes maurus*), and in the south of the peninsular, the betic midwife toad (*Alytes dickhilleni)*, are restricted almost completely to mountainous areas suitable for the development of the disease. Sampling carried out in Morocco determined the presence of *Bd*, for the first time in the north of the African continent, in several species and different areas of the country (Chapter 2) and, although infection was not detected at *A. maurus*, more recent surveys have confirmed *Bd* mortality in the species.

In the south of the Iberian Peninsula, the samplings carried out throughout the distribution of *A. dickhilleni* detected the presence of *Bd* in two very distant areas, with a prevalence of 100%, suggesting a recent introduction with little dispersion. Although no mortality was detected in the field, it was higher than 70% in the laboratory, confirming the risk of *Bd* expansion for the species (Chapter 3). In fact, in recent years, *Bd* has been spreading throughout the range of the betic midwife toad. The first mass mortalities have been recorded in several populations that were key to the species, leaving them near of extinction.

In parallel, we monitored all existing populations of *Calotriton arnoldi*, the Montseny newt, classified as endangered in the Spanish Catalogue of Threatened Species, searching for the presence of *Bd,* although in this case, with negative results in all samples (Chapter 4).

Environmental conditions entirely determine the impact of *Bd* on amphibian populations; so one of our first objectives was to assess the influence of temperature, *a priori,* the main abiotic factor involved in the dynamic of the disease. We showed that low temperatures are, in short-term, the best predictor of infection intensity in populations of infected *A. obstetricans* in low altitude areas of Zamora (Chapter 5). After an exhaustive monitoring of six populations during a full year and recording both the water temperature and the infection intensity of the larvae, we obtained a marked seasonal pattern. Infection was inversely correlated with temperature, with the highest values (both intensity and prevalence of infection) in the coldest months of the winter. These results could be explained by the lack of ability larvae to cope with the infection under extreme environmental conditions, rather than the suitability of these

conditions for the growth of the fungus, given that the recorded temperatures were outside of the optimal range of the pathogen *in vitro*.

In Sierra de Guadarrama National Park, the rapid and severe decline suffered by the most affected species, the common midwife toad (*Alytes obstetricans*), led to the implementation of a captive breeding program. For the establishment of the breeding colony, we captured some of the few surviving specimens of the epidemic. At the same time, we carried out a genetic study using molecular markers (microsatellites) in the remaining reproductive populations to verify the suitability of these specimens to be used as a parents of the breeding program and to design the most appropriate crosses (Chapter 6). As a result of the analysis of the population's genetic structure of *A. obstetricans* in the Peñalara massif, we observed low genetic variability with clear evidences of a strong bottleneck as a consequence of the disease. This fact, together with the relatively low amphibian dispersal capacity and the presence of geographic barriers, was associated with the strong genetic structure observed in the population. Therefore, in order to recover the original genetic diversity and to avoid a possible inbreeding depression, we recommended using a mixture of specimens from different locations as a source of organisms for future reintroductions. The specimens of all remnant-breeding points can be considered as a single population, except for the breeding pond that is most geographically distant, because it seems that there is no genetic exchange with that site.

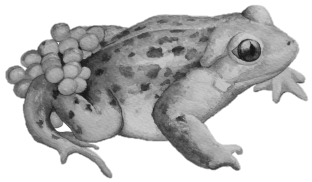
It has been shown that the incidence of chytridiomycosis varies greatly between species. In order to evaluate the role of each species within the amphibian community in the transmission and dynamics of the disease, we carried out experimental infections in the laboratory and field experiments. The results corroborate the role of *A. obstetricans* as the main reservoir of infection and reflect how their presence is responsible for the increased burden of zoospores on other species by direct transmission behaving, therefore, as a super-host. In addition, we showed the different susceptibility to infection in all species studied (Chapter 7).

The near disappearance of the overwintering larvae of *A. obstetricans* from the Peñalara lagoons led to the expansion of *Bufo spinosus*, the common spiny toad, by occupying the permanent lagoons that had left empty. The monitoring to verify and quantify this phenomenon was carried out using capture/recapture methods in several sampling sessions. We introduced more than 1.500 individual microchips throughout 5 years to

analyse the demographic parameters of the population (Chapter 8). In accordance with what was observed at the field (high mortality at the shores of the lagoons at the time of metamorphosis), the results confirm a low recruitment rate (lower than that estimated for other toads living in the same environment) that is not sufficient to compensate for the survival rate of the species, condemning the population to a slow, but inexorable decline.

As can be deduced from the previous paragraph, *Bd* is able to alter the structure of an entire amphibian community due to its ability to infect different hosts with variable susceptibility to infection. The implementation of long-term monitoring programs to record changes in the population trends of the different species has proved to be a very useful way of knowing what has happened in the Peñalara Massif after the *Bd* incursion (Chapter 9). Since 1999, the year in which the monitoring program was initiated, an intensive study of population trends has been developed, doing up to 6 visits throughout the breeding season to the 29 sectors of catalogued water points. In these visits larval and/or clutches' counts (depending on the species) were undertaken in the nearly 250 ponds and lagoons catalogued and assigned to the different established categories of abundance. The analysis of population trends reveals that only 3 species have suffered significant declines, while other species remain stable or even have experienced population growth related to environmental changes.

This thesis reveals, on the one hand, the enormous complexity we face in the fight against this pathogen and, on the other, the need for multifactorial approaches to address its study. To better understand the dynamics of the disease, there are key factors that must be taken into account, *i.e.*, the ability of *Bd* to spread rapidly through various vectors, the intensity of the declines it produces, the wide range of hosts that it is capable of infecting, the different susceptibility of the hosts and finally, but not less important, the influence of environmental variables. Conclusively, this thesis sets a precedent in the analysis of the incidence of emerging diseases, describing the basic lines of study in other new pathogens with potentially harmful consequences for biodiversity.



CAPÍTULO 1. INTRODUCCIÓN

APORTACIONES AL CONOCIMIENTO DEL IMPACTO DE *BATRACHOCHYTRIUM DENDROBATIDIS* EN ESPAÑA

APORTACIONES AL CONOCIMIENTO DEL IMPACTO DE *BATRACHOCHYTRIUM DENDROBATIDIS* EN ESPAÑA

A las múltiples amenazas que han afectado a los anfibios ibéricos durante las últimas décadas, en épocas recientes se ha sumado la quitridiomicosis. El agente causal de esta enfermedad emergente es el hongo *Batrachochytrium dendrobatidis*, también conocido como el "hongo asesino", por afectar a más de 700 especies en todo el mundo y ser responsable del declive y la extinction de muchas de ellas (Lips 2016). Este nuevo género del orden Chytridiales fue descrito formalmente en 1999 (Longcore *et al.* 1999), siendo el primer y único caso conocido de infección por hongos quitridios en vertebrados. El hongo se reproduce por zoosporas acuáticas que son liberadas al medio e infectan tanto a las larvas como a los adultos, aunque las mortalidades masivas en climas templados suelen suceder al final de la metamorfosis (Bosch *et al.* 2001, Garner *et al.* 2009). En general, prefiere temperaturas frescas y detiene su crecimiento, e incluso muere, a altas temperaturas (Piotrowsky *et al.* 2004). *Batrachochytrium dendrobatidis* (en adelante *Bd*) ha sido detectado ya en más de 50 países y, preocupantemente, los mayores declives conocidos se han producido en zonas tropicales con alta diversidad de anfibios, pero también en áreas de montaña bien conservadas en zonas templadas (revisión en Lips 2016). Estos requerimientos ambientales del patógeno hacen de los sistemas montañosos ibéricos lugares idóneos para la implantación y la permanencia del hongo y así ha sucedido desde finales de los años 90 hasta la actualidad.

¿Qué impacto produce Bd en las zonas templadas?

Desde los primeros registros de mortalidades masivas por *Bd* en Australia y Centroamérica, mucho hemos aprendido sobre su biología y sobre la dinámica de la enfermedad. Sin embargo, en los veranos de 1997, 1998 y 1999 detectamos las primeras mortalidades masivas de ejemplares recién metamorfoseados de *Alytes obstetricans* en las lagunas del Macizo de Peñalara, corazón del Parque Nacional de la Sierra de Guadarrama (Fig. 1). En ese momento, no podíamos imaginar que un problema aparentemente circunscrito a zonas tropicales (Berger *et al.* 1998, Daszak *et al.* 1999, Pessier *et al.* 1999) pudiera tener un impacto tan severo en las zonas templadas del planeta. Se trataba del primer caso de declive de anfibios conocido en Europa como consecuencia de la enfermedad (Bosch *et al.* 2001) y el primer

indicio de que, también en las zonas templadas, la quitridiomicosis podía producir extinciones locales y declives superiores al 90%.

Figura 1. Laguna de Pájaros en el Macizo de Peñalara, Parque Nacional de la Sierra de Guadarrama. Uno de los pocos núcleos de *A. obstetricans* que resistió a la epidemia de quitridiomicosis, aunque pasando de contar con más de 5.000 larvas a menos de 10.

Enseguida nos preguntamos si lo que estaba pasando se circunscribía sólo a la Sierra de Guadarrama y pusimos en marcha una serie de muestreos por toda la Península Ibérica y otras zonas de Europa, en colaboración con colegas de otros países. La respuesta fue clara: *Bd* ya estaba ampliamente distribuido por toda Europa y podría afectar a un gran número de especies, aunque de manera irregular, siendo excepcionalmente alta la prevalencia de la infección en Suiza y España (Garner *et al.* 2005). Curiosamente, éstos eran los países europeos con mayor altitud media, lo que inducía a pensar que en zonas templadas, las áreas de montaña presentaban las condiciones climáticas más favorables para el desarrollo del patógeno.

Aunque hasta ese momento la enfermedad sólo había sido relacionada con mortalidades masivas en Australia y Centroamérica, los declives "misteriosos" de anfibios se sucedían en todo el mundo desde hacía décadas. De hecho, un tercio de las casi 7.000 especies conocidas de

anfibios están catalogadas como en peligro, convirtiendo a los anfibios en el grupo más amenazado de todos los vertebrados terrestres debido a los múltiples factores que amenazan sus poblaciones y que actúan, además, de forma sinérgica (Stuart *et al.* 2004, 2008). La incorporación de muchas localidades de la península ibérica y de Europa como puntos favorables para el desarrollo de la quitridiomicosis, amplió el rango de los modelos de nicho ecológico que se habían realizado hasta la fecha (Ron 2005) y nuestros nuevos modelos resultaron aún más alarmantes (Rödder *et al.* 2009). Casi toda España y grandes zonas del continente europeo se mostraban como zonas muy favorables para el desarrollo de la infección, y hasta un sexto del total de especies de anfibios analizadas, concretamente 379, presentaban su distribución total en zonas sensibles a *Bd*, ampliándose el número de especies potencialmente vulnerables y que deberían ser objeto de atención especial (Rödder *et al.* 2009).

Además, con la incorporación de grandes zonas de Europa como hábitats susceptibles al desarrollo de la enfermedad, resultaba ya posible establecer una conexión clara entre aquellos declives catalogados como "enigmáticos" décadas atrás en varias regiones del mundo y la quitridiomicosis (Lötters *et al.*, 2009). El problema empezaba a adquirir grandes dimensiones y, sin embargo, aún sabíamos muy poco de por qué la incidencia de la enfermedad era tan desigual entre especies y entre localidades.

Así, nos centramos de nuevo en Peñalara, y comprobamos que tras el brutal declive de *A. obstetricans*, al menos otras dos especies (*Bufo spinosus* y *Salamandra salamandra*) comenzaron a sufrir mortalidades masivas (Bosch y Martínez-Solano 2006). En otras zonas de la Península Ibérica, los nuevos brotes que registramos en Pirineos, la Serra da Estrela y Mallorca tenían al género *Alytes* como protagonista indiscutible (Fig. 2; Walker *et al.* 2008, 2010, Rosa *et al.* 2013). Curiosamente, las tres especies más seriamente afectadas presentaban hábitos eminentemente terrestres, cuando en los trópicos las especies más susceptibles a *Bd* eran aquellas más ligadas al medio acuático. Sin embargo, las tres especies, y especialmente *Alytes*, presentan estadios acuáticos prolongados, incluso con larvas invernantes en el caso de *Alytes* y *Salamandra* en las zonas de montaña, y las mortalidades masivas se registraban en masas de agua permanentes donde la temperatura del agua nunca es demasiado elevada.

Figura 2. Ejemplares recién metamorfoseados de *Alytes obstetricans* en Pirineos muertos por quitridiomicosis.

Sin embargo, el impacto de la enfermedad en las tres especies resultó muy desigual. Parecía que el elevado tamaño de puesta y el desarrollo larvario restringido a una estación de *B. spinosus* podría hacerle mucho más resistente a la enfermedad. Sin embargo, cada año gran cantidad de ejemplares recién metamorfoseados aparecían muertos alrededor de las lagunas y, sorprendentemente, muchos de ellos resultaban negativos para *Bd* tras ser analizados en el laboratorio (Fig. 3). Lamentablemente, nuestras observaciones de campo y experimentos de infección en laboratorio sugerían que la lucha contra el hongo conlleva un coste muy elevado, en términos de crecimiento y deterioro de condición corporal, que acaba traduciéndose en mortalidad, incluso aunque el sistema inmunológico del ejemplar consiga terminar con el patógeno (Garner *et al.* 2009). Así, el impacto de *Bd* sobre las poblaciones de *B. spinosus* de Peñalara quedó patente cuando, años más tarde, analizamos sus parámetros demográficos mediante el marcaje y seguimiento individual de más de 1.500 ejemplares durante 5 años (Bosch *et al.* 2014). En la laguna Grande de Peñalara, al contrario que en la Laguna Chica donde su carácter temporal suaviza la incidencia de *Bd*, se produce una elevada mortalidad de *B. spinosus* tras la metamorfosis. Esta baja tasa de reclutamiento, menor que la observada en otros sapos de ambientes similares (Muths *et al.* 2011), no es suficiente para

compensar la probabilidad de supervivencia de la especie y, por tanto, condena a la población a un lento, pero continuo declive (Bosch *et al.* 2014).

Figura 3. Mortalidad de *Bufo spinosus* por quitridiomicosis en la Laguna Grande de Peñalara.

Una vez que sabíamos que la lucha de los hospedadores contra el patógeno conlleva un coste, se hacía importante poder medirlo de alguna forma. Para ello, desarrollamos un nuevo método de análisis no invasivo que nos permitía recoger la cantidad de hormona corticosterona secretada por los organismos, pudiendo evaluar así su nivel de estrés (Gabor *et al.* 2013a). Efectivamente, pudimos comprobar en larvas del género *Alytes* que los ejemplares infectados presentaban unos niveles de estrés muy superiores

a los no infectados (Gabor *et al.* 2013b), lo que acabaría probablemente traduciéndose en afecciones al sistema inmune. Las defensas inmunológicas innatas de la piel de los anfibios podrían ser la principal protección ante la infección por *Bd*, especialmente por la producción de péptidos antimicrobianos y por la reacciones inflamatorias de la piel y, aunque aún no ha podido ser demostrado, es posible que estas reacciones incrementen aún más la vulnerabilidad de los ejemplares.

Pero *Bd* no sólo produce un elevado coste sobre los individuos o incluso su muerte, sino que ocasiona importantes alteraciones en las comunidades de anfibios. Así, la enfermedad podría estar cambiando la estructura de las comunidades, alterando las interacciones bióticas entre ellas por la diferencia de susceptibilidad entre las especies. En Peñalara, y pese a los profundos efectos adversos de *Bd* sobre *B. spinosus* descritos, fuimos testigos de la expansión de la especie tras la casi completa extinción de *A. obstetricans* (Bosch & Rincón 2008). Experimentalmente demostramos que *B. spinosus* evita los lugares de puesta con presencia de larvas de *A. obstetricans*, quizás como consecuencia de la reducción en la condición corporal, crecimiento y supervivencia de sus larvas cuando entran en competencia con las de *A. obstetricans* (Richter-Boix *et al.* 2007). En Peñalara vimos como la desaparición de las grandes larvas invernantes de *A. obstetricans* por *Bd*, motivó la expansión de *B. spinosus* al ocupar las lagunas permanentes que habían quedado vacías, sin sospechar que, años más tarde, se convertirían en una trampa mortal para los ejemplares recién metamorfoseados.

¿Por qué el impacto de Bd es tan variable entre especies y localidades?

Bd es un patógeno generalista capaz de infectar multitud de especies de anfibios, pero a la vez presenta enormes diferencias en patogenicidad, y no sólo relacionadas con la susceptibilidad del hospedador, como parecía inicialmente. Así, una compleja red de interacciones entre factores bióticos y abióticos modulan el desarrollo de la enfermedad, y se hace necesario conocer cómo se comportan los diferentes hospedadores, el papel que juegan los diferentes estadios de desarrollo y la enorme influencia del ambiente.

A lo largo de estos años hemos ido esclareciendo cómo funcionan algunas de estas interacciones y corroborando ciertas hipótesis. Hoy sabemos que todas las especies de Peñalara son susceptibles de ser infectadas por *Bd*,

pero que el grado de susceptibilidad varía enormemente de unas a otras (Fernández-Beaskoetxea *et al.* 2016). La intensidad de la infección varía mucho en función del estadio de desarrollo de los individuos, siendo la metamorfosis el momento más crítico al coincidir la expansión de la queratina por toda la piel del ejemplar con la supresión del sistema inmune. Pero también la condición corporal de los individuos resulta clave para superar con éxito la infección (Garner *et al.* 2009), por lo que también es importante considerar todo lo que sucede en los estadios larvarios que son susceptibles de albergar la infección y juegan un papel clave como reservorios y transmisores de la enfermedad.

Claramente, *Alytes* es el taxón más vulnerable a la enfermedad en Europa, pero ahora también sabemos que sus larvas, además, se comportan como super-hospedadores que mantienen y amplifican la infección en otras especies por transmisión directa (Fernández-Beaskoetxea *et al.* 2016). Como vimos antes, su largo periodo larvario en zonas de montaña (hasta 5 años en Peñalara, García-París, comunicación personal) y su gran disco oral con abundante queratina podrían resultar claves.

Pero, sin duda, la existencia de larvas invernantes resulta determinante para el mantenimiento y la amplificación de la infección, como pudimos comprobar mediante el estudio de las larvas invernantes de *S. Salamandra* (Medina *et al.* 2015). Así, las interacciones entre el tipo de hábitat (charcas frente arroyos), el estadio de desarrollo (larvas invernantes frente a larvas del año) y el hidroperiodo de las masas de agua (puntos de agua permanentes frente a temporales) resultaron claves para explicar la incidencia de *Bd* en las larvas de *S. salamandra*, que se han convertido en el principal reservorio de la infección tras la desaparición de *A. obstetricans* en Peñalara. En los arroyos, las zoosporas de *Bd* son arrastradas por la corriente, por lo que la transmisión es menor y las cargas de infección son más bajas. En las charcas, la transmisión del patógeno es más fácil, pero las masas de agua temporales se calientan mucho en verano y no permiten infecciones elevadas. Sólo en las charcas permanentes, la ausencia de temperaturas elevadas hace que los niveles de infección se mantengan elevados en las larvas invernantes, que mantienen la infección todo el año y transmiten el patógeno a las nuevas larvas del año nacidas en primavera. Así, las larvas de arroyos y de charcas temporales no sucumben al patógeno y mantienen la población de *S. salamandra* de Peñalara, mientras que muchas larvas invernantes de charcas permanentes mueren al completar la metamorfosis, permitiendo a la vez la persistencia de *Bd* en el sistema.

Pero no sólo ciertos factores bióticos de los hospedadores moldean la dinámica de la quitridiomicosis, sino que el propio patógeno está también sujeto a factores intrínsecos. Al tratarse de una enfermedad emergente producida por un organismo claramente invasor, al principio no se prestó mucha atención a la variación del patógeno. Sin embargo, cuando comparamos la morfología de diferentes cultivos de la Península Ibérica con otros de Mallorca y de Inglaterra, encontramos variaciones morfológicas importantes como el tamaño del esporangio y que, de forma reveladora, aparecían ligadas a distintos niveles de patogenicidad (Fisher *et al.* 2009). Este hecho abrió la puerta a estudios genéticos complejos del hongo y culminó con la descripción de diversos linajes genéticos de *Bd* que ha resultado clave para entender la incidencia de la enfermedad en el mundo. Efectivamente, *Bd* no es homogéneo a lo largo y ancho de toda su área de distribución. No obstante, la forma más generalizada, y también la más patogénica que se encuentra en continua expansión, es la que denominamos GPL, de sus siglas en inglés, 'Global Panzootic Lineage', que habría surgido por recombinación genética de linajes previamente alopátricos que habrían entrado en contacto mediante movimientos artificiales de anfibios por el ser humano (Farrel *et al.* 2011).

Inmediatamente, se puso de manifiesto la influencia de ciertos factores abióticos sobre el desarrollo de la infección. Hoy sabemos, por ejemplo, que la prevalencia de la infección en *A. obstetricans* está inversamente correlacionada con la cantidad de radiación UV y que el riesgo de mortalidad aumenta drásticamente en áreas con bajas temperaturas mínimas y elevada altitud (Bosch *et al.* 2007, Walker *et al.* 2010 y Ortíz-Santaliestra *et al.* 2011). Hasta hace relativamente poco tiempo, se pensaba que la radiación UV podría agravar el efecto de la quitridiomicosis al hacer a los anfibios más vulnerables a la enfermedad por el debilitamiento de su sistema inmune (revisión en Tevini 1993). Esta idea resultaba razonable teniendo en cuenta que muchos declives de anfibios se habían registrado en regiones montañosas de todo el mundo, que se había constatado que *Bd* podía desarrollarse por encima de los 5.000 m de altura y que la radiación UV parecía no afectarle en experimentos de laboratorio (Johnson *et al.* 2003). Sin embargo, al contrario de lo esperado, cuando analizamos experimentalmente la influencia de la radiación UV en la infección de larvas de *B. spinosus* en Peñalara, observamos una prevalencia más baja en las larvas expuestas a la radiación que en las que estaban protegidas de la misma (Ortíz-Santaliestra *et al.* 2011), coincidiendo con lo que obtuvimos en poblaciones naturales de *A. obstetricans* de la Península Ibérica (Walker *et al.* 2010). Esto es, la radiación UV destruye las zoosporas

y contribuye, por tanto, a controlar la infección en zonas altas, aunque su efecto está supeditado a otros factores, como veremos más adelante.

Pero sin duda, el factor abiótico que más contribuye al desarrollo de la enfermedad es la temperatura. Así, la temperatura modula, por un lado, la capacidad de respuesta del hospedador a la infección y, por tanto, su susceptibilidad y, por otro lado, la tasa de crecimiento y desarrollo del patógeno (Woodhams *et al.* 2003, Berger *et al.* 2004, Ribas *et al.* 2009).

Cuando tuvimos ocasión de estudiar el efecto de *Bd* sobre las poblaciones de *A. muletensis* en Mallorca, pudimos comprobar la enorme influencia de la temperatura sobre el desarrollo de la enfermedad (Fig. 4). Dos poblaciones muy próximas de la isla estaban infectadas con una prevalencia en torno al 100% en invierno (Walker *et al.* 2008). Sin embargo, mientras que la población natural (el Torrent des Ferrerets) casi llegó a desaparecer completamente tras la introducción del hongo, la otra población (el Cocó de Sa Bova) no ha dejado de crecer desde que fue creada con el programa de reintroducción de la especie. La diferencia entre ambas poblaciones radica en su régimen de temperaturas, que regula completamente las dinámicas ente el patógeno y el hospedador. Así, las pozas del Cocó de Sa Bova están totalmente expuestas a la insolación y las temperaturas del agua se mantienen casi todo el tiempo por encima de los 25ºC, una temperatura suficiente para disminuir el crecimiento del hongo. Sin embargo, en el Torrent des Ferrerets, la ausencia de insolación directa en las pozas hace que las temperaturas se mantengan muy estables durante todo el año, y nunca superen los 20ºC, lo que aumentaría la producción de zoosporas y su periodo con capacidad de infección (Doddington *et al.* 2013). Por lo tanto, incluso a una escala geográfica de escasos kilómetros, las condiciones ambientales determinan completamente el impacto de *Bd* sobre las poblaciones de anfibios.

De hecho, son las temperaturas bajas, y a una corta escala temporal, el mejor predictor de la intensidad de la infección, tal y como observamos en poblaciones de *A. obstetricans* a baja altitud infectadas en Zamora (Fernández-Beaskoetxea *et al.* 2015). Tras realizar un seguimiento exhaustivo de seis poblaciones durante un año completo registrando tanto la temperatura del agua como el grado de infección de las larvas, obtuvimos un marcado patrón estacional. La infección se correlacionaba inversamente con la temperatura, presentando sus valores más altos (tanto en intensidad como en prevalencia de infección) en los meses más fríos del invierno. Como dijimos antes, estos resultados podrían explicarse por la falta de

capacidad de las larvas para hacer frente a la infección bajo condiciones ambientales extremas, y no tanto a la patogenicidad del hongo, ya que las temperaturas registradas se encontraban fuera del rango de crecimiento óptimo del patógeno *in vitro*.

Figura 4. Ejemplar adulto de *Alytes muletensis* muerto por quitridiomicosis en un torrente de la Sierra de Tramuntana.

Pero la naturaleza siempre es más compleja y rara vez el estudio de un solo factor puede explicar completamente la dinámica de la infección en un determinado lugar. Así, uno de nuestros últimos estudios en Peñalara ha revelado que existen complejas interacciones entre factores bióticos y abióticos, concretamente la radiación UV, el zooplancton y la composición de las comunidades de anfibios, enmascarando el efecto del hábitat sobre la prevalencia de infección (Hite *et al.* 2016). Por un lado, los experimentos de campo demostraron que la radiación UV, efectivamente, elimina gran cantidad de zoosporas. Sin embargo, los puntos de agua permanentes que reciben mayor radiación presentan también los niveles más altos de infección. Esta paradoja se resuelve por la existencia de dos efectos indirectos que trabajan juntos para compensar la pérdida de zoosporas por la radiación UV: la presencia de larvas invernantes en los puntos de agua permanentes, que hacen proliferar la infección, y la reducción de la cantidad de zooplancton debido a la radiación, que es un importante

depredador de las zoosporas. Para complicar aún más el sistema, la diversidad de especies hospedadoras, que no está relacionada con el hidroperíodo ni con la radiación, actúa contrarrestando la infección por el llamado efecto "dilución" (Searle *et al.* 2011) y además, la presencia de urodelos favorece la infección, ya que sus larvas depredan sobre el zooplancton, depredador a su vez de zoosporas de *Bd* (Hite *et al.* 2016).

¿POR QUÉ *BD* ESTÁ ACTUANDO AHORA?

Existen dos grandes hipótesis, no mutuamente excluyentes, para explicar el creciente impacto de *Bd* sobre las poblaciones de anfibios. La "hipótesis del patógeno introducido" postula que *Bd* es una especie introducida que ha ido expandiéndose mostrando el típico frente epidémico (Lips *et al.* 2006). Esta hipótesis recibió el soporte de los primeros estudios de genética de poblaciones (Daszak *et al.* 2003, Morehouse *et al.* 2003) y del hecho, de que existen especies invasoras muy extendidas por el ser humano que portan la enfermedad de manera asintomática, como la rana toro americana, *Lithobates catesbeianus* (Garner *et al.* 2006). Por otro lado, hace ya algunos años, algunos investigadores vieron en la "hipótesis del patógeno endémico" la única forma sensata de explicar la repentina aparición de la enfermedad en todo el mundo. Esto es, *Bd* siempre habría estado en las zonas afectadas por la enfermedad, pero un cambio en las condiciones climáticas habría favorecido su desarrollo (Pounds *et al.* 2006). Sin embargo, todos los estudios genéticos que vinieron después de esos años apoyaron la primera hipótesis (e.g., Morgan *et al.* 2007, James *et al.* 2009). En España, nuestro estado de conocimiento de la situación nos llevó a buscar relaciones entre las condiciones ambientales y la intensidad de la infección en 126 poblaciones de *A. obstetricans* (Walker *et al.* 2010). Los resultados fueron claros: como ya hemos visto, las condiciones ambientales modulaban completamente el desarrollo de la enfermedad, tal y como establece la hipótesis del patógeno endémico, sin embargo, el patrón observado de presencia del hongo era consistente con la hipótesis del patógeno introducido, es decir, las características ambientales no explicaban la presencia de este patógeno generalista que está presente, prácticamente, allí donde ha podido llegar.

Sin embargo, y como también vimos antes, el hecho de tratarse de un patógeno introducido no significa que no pueda mostrar una considerable diversificación, sobre todo si tenemos en cuenta que 1) se trata de un organismo con capacidad de evolucionar rápidamente, 2) su expansión por todo el mundo podría haber sido anterior a lo que imaginábamos y 3) el

linaje globalmente distribuido y altamente patogénico convive con linajes autóctonos en varias regiones del planeta.

De hecho, documentar la llegada de *Bd* a un territorio concreto no es fácil. Mallorca es, precisamente, uno de los pocos lugares en el mundo para el que tenemos esta certeza: tristemente, *Bd* fue introducido en la isla a través del programa de reintroducción de *A. muletensis* (Walker *et al.* 2008). Tras la aparición del primer ejemplar recién metamórfico encontrado muerto en 2004 en Mallorca y la confirmación de que estaba infectado con *Bd*, analizamos todas las poblaciones existentes en la naturaleza, así como la historia de las reintroducciones realizadas a través del plan de recuperación de la especie en la década de 1990. Por desgracia, los ejemplares criados en el Zoo de Jersey, y que fueron liberados en Mallorca logrando un éxito sin precedentes en la recuperación de un anfibio gravemente amenazado, habían estado en contacto estrecho con ejemplares de *Xenopus gilli* de Sudáfrica. Tras la llegada de estos ejemplares a Jersey en 1991, cinco de ellos murieron junto con otros 23 *A. muletensis* de la colonia cautiva, sin que nadie supiese explicar el motivo. Varios de estos ejemplares muertos y preservados en formol en la década de 1990 darían positivo para *Bd* cuando los analizamos, y esto explica que Mallorca sea el único lugar en el mundo donde se conoce la presencia de la cepa de *Bd* del Cabo, presente en Sudáfrica. Afortunadamente, esta cepa es menos virulenta que la GPL, y la infección quedó relegada sólo a las dos localidades que veíamos antes: el Torrent des Ferrerets y el Cocó de Sa Bova.

La especial susceptibilidad de las especies del género *Alytes*, así como la de otras especies endémicas, con distribuciones restringidas y localizadas en zonas favorables al desarrollo de la quitridiomicosis, nos impulsó a analizar la situación en otras áreas geográficas. En el norte de Marruecos, *Alytes maurus*, y en el sur Peninsular, *Alytes dickhilleni*, están restringidos casi completamente a zonas montañosas idóneas para el desarrollo de la enfermedad. Nuestros muestreos en Marruecos determinaron la presencia de *Bd*, por primera vez en el norte del continente africano, en varias especies y distintas zonas del país (El Mouden *et al.* 2011) y, aunque en ese momento no constatamos la infección en *A. maurus*, muestreos más recientes han confirmado mortalidad por *Bd* en la especie (D. Donaire and J. Bosch, datos no publicados). En el sur peninsular, nuestros muestreos llevados a cabo en toda la distribución del sapo partero bético (*A. dickhilleni*) detectaron la presencia de *Bd* en dos zonas muy alejadas entre si, y con una prevalencia del 100%, sugiriendo una introducción reciente con escasa dispersión (Bosch *et al.* 2013). Aunque en ese momento no detectamos

mortalidad en el campo, ésta fue superior al 70% en laboratorio, confirmándose el riesgo que supondría la expansión de *Bd* para la especie. La emergencia de *Bd* en esta especie seguía un patrón parecido al que observamos en Mallorca. Las dos únicas poblaciones infectadas se relacionaban con la actividad naturalista o investigadora, lo que ponía de manifiesto, una vez más, nuestra enorme responsabilidad como naturalistas para evitar la expansión del patógeno (Bosch *et al.* 2013). En los últimos años, *Bd* se está extendiendo por toda su área de distribución, y las primeras mortalidades masivas registradas han dado paso a la casi total extinción de varias poblaciones que resultaban clave para la especie (J. Bosch *et al.* datos no publicados). A lo largo del mismo año, también llevamos a cabo muestreos en todas las poblaciones existentes de *Calotriton arnoldi,* el tritón del Montseny, aunque en este caso, con resultados negativos en todas las muestras (Obón *et al.* 2013).

Como veíamos antes, el hecho de que estemos ante un patógeno introducido no significa que no puedan existir cambios ambientales que puedan exacerbar la enfermedad. Así, y dado que las áreas colonizadas por el patógeno tienen que cumplir unos requerimientos concretos para que se alcance su óptimo de crecimiento y produzca un impacto evidente, la hipótesis de la epidemia ligada a cambios ambientales o del patógeno endémico ha sido rebautizada como "hipótesis del óptimo térmico del hongo" (Pounds *et al.* 2006). En Peñalara, nuestros análisis de los datos meteorológicas durante 28 años, antes y después de la aparición de los primeros brotes de la enfermedad (Bosch *et al.* 2007) revelaron una asociación significativa entre ciertos cambios en las condiciones climáticas locales y la incidencia de la quitridiomicosis. El aumento de la temperatura y de la humedad en la zona fue notable justo antes de la epidemia, registrándose un aumento significativo en el número de días soleados y cálidos en julio y agosto, cuando tiene lugar la metamorfosis y los anfibios son más vulnerables a *Bd*. Así, los efectos del calentamiento global registrados en la zona estarían aumentando el número de días con temperaturas que se encuentran dentro del óptimo de desarrollo del patógeno sin que, por supuesto, las temperaturas alcancen los valores elevados que resultan ya críticos para *Bd*. Por tanto, el calentamiento global de las zonas templadas, más notable en zonas de montaña, podría estar exacerbando la enfermedad en zonas altas, acercando la temperatura del medio al óptimo de crecimiento del patógeno. Por otro lado, el cambio climático también podría estar relacionado con la expansión del hongo hacia zonas altas de montaña, pues también en Peñalara hemos podido registrar la expansión en altura de algunas especies más propias de zonas

bajas que podrían haberse comportado como vectores de *Bd* (Bosch *et al.* datos no publicados).

Otro efecto notable producido por el calentamiento global en zonas de montaña es el adelanto de la fecha de deshielo. Nuestros trabajos en Pirineos revelaron la implicación de este fenómeno en la intensidad de infección de tres especies que están sufriendo declives poblacionales como consecuencia de la quitridiomicosis: *Rana temporaria* (la especie local más resistente a la infección), *B. spinosus* (con una tolerancia media a la infección) y *A. obstetricans* (extremadamente susceptible). Tras más de una década trabajando en la zona, pudimos comprobar cómo el adelanto en la fecha de deshielo en la zona aumenta enormemente el grado de infección por *Bd* en las dos especies menos vulnerables, mientras que *A. obstetricans* presentaba siempre una elevada prevalencia independientemente del momento del deshielo (Clare *et al.* 2016).

¿QUÉ PODEMOS HACER ANTE ESTA SITUACIÓN?

Recientes investigaciones que hemos desarrollado también en Peñalara han demostrado, de forma esperanzadora, que el componente heredable asociado a la infección por *Bd* parece ser lo suficientemente alto como para que pueda existir una adaptación al patógeno (Palomar *et al.* 2016). No obstante, este proceso, si llega a producirse, será largo. Y mientras sucede, muchas poblaciones de las especies altamente sensibles, y quizás incluso algunas de nuestras especies endémicas, desaparecerán. Por lo tanto, no podemos quedarnos con los brazos cruzados y debemos enfocar todas nuestras investigaciones al desarrollo de métodos paliativos de la enfermedad.

En nuestra primera revisión sobre las posibles medidas de mitigación de la enfermedad (Woodhams *et al.* 2011) establecimos las prioridades de manejo, que se concentran en frenar la expansión del patógeno, mantener colonias cautivas seguras de las especies y poblaciones más vulnerables y desarrollar métodos de profilaxis o remedios contra la enfermedad. Sin embargo, todo intento de mitigación debe ajustarse a las condiciones ambientales para disminuir la susceptibilidad de los anfibios y/o la patogenicidad de *Bd* y no debemos centrarnos únicamente en la erradicación del patógeno o en la cría en cautividad, sino proponer tratamientos a nivel poblacional. Para ello, es necesario primero, identificar los mecanismos de supresión de la enfermedad y después, parametrizar las variables que regulan la infección mediante modelos testados en

condiciones naturales para, finalmente, emprender procesos de manejo adaptativos en el campo en condiciones naturales.

Transcurridos algunos años desde el establecimiento de estas recomendaciones y aunque las actuaciones de conservación *ex situ* siguen siendo necesarias, son muy escasos los programas de investigación que han ensayado actuaciones *in situ* en el mundo (Garner *et al.* 2016). Precisamente en Mallorca, el hecho de tratarse de un sistema insular, con un único hospedador, unido al ambiente extraordinariamente seco que presenta la isla y a la altísima probabilidad de que el patógeno acabe llegando a todas las poblaciones a través de los barranquistas, nos animó a emprender un intento de erradicación del patógeno pionero en el mundo (Bosch *et al.* 2015). El trabajo, de más de cinco años, utilizando antifúngicos en laboratorio para el tratamiento de todas las larvas existentes al final del verano y el posterior secado de las pozas, recibió una atención mediática considerable (Naomi Lubik 2010), pero culminó sin éxito con la reinfección de las poblaciones tratadas. Sin embargo, la inminente extinción local de la población del Torrent des Ferrerets (aquel de aguas frías que era una de las pocas localidades naturales donde la especie consiguió resistir), junto con la autorización de la Consejería de Medio Ambiente del Gobierno Balear, nos animó a la aplicación de un desinfectante químico en el medio una vez retirados los animales y vaciadas las pozas. De esta forma, la infección fue erradicada en cuatro de los cinco sitios tratados, y se mantuvo así durante varios años después de la aplicación. No fue el fin de la guerra contra la quitridiomicosis, pero si un batalla ganada que demuestra que actuaciones sencillas, aunque costosas, pueden y deben llevarse a cabo mientras no contemos con otras soluciones más definitivas (Bosch *et al.* 2015).

Nuevas amenazas

Paralelamente al avance en el conocimiento de los mecanismos que regulan la dinámicas de la quitridiomicosis, nuevas amenazas surgen para los anfibios. Una nueva especie de hongo quitridio, *Batrachochytrium salamandrivorans* (*Bsal*), originario de Asia, ha llegado ya al norte de Europa probablemente a través del comercio internacional de mascotas como los tritones del género *Cynops*. Su presencia restringida en Europa, unas pocas localidades entre Holanda, Bélgica y Alemania, ha provocado ya declives dramáticos en *S. salamandra* del norte de Europa (Martel *et al.* 2013, Spitzen-van der Sluijs *et al.* 2013). Nuestro análisis de más de 5.000 muestras de toda Europa y las infecciones experimentales en laboratorio de

un número importante de especies de anfibios confirman que aún no se ha extendido desde su zona de introducción, pero también que se trata de otro hongo altamente patógeno para la gran mayoría de las especies de urodelos (Martel *et al.* 2014). Su inevitable llegada a la Península Ibérica, supondrá un nuevo reto en la conservación de nuestra herpetofauna, que debería tomar ventaja de todas nuestras experiencias y conocimientos adquiridos con *Bd*.

Por último, otro viejo patógeno conocido como causantes de mortalidades masivas de anfibios, *Ranavirus*, ha cobrado relevancia recientemente. Cuando en 1992 se detectaron miles de larvas muertas de *A. obstetricans,* en un ibón del Pirineo oscense (Márquez *et al.* 1995), con evidentes síntomas de hemorragias e inflamaciones generalizadas, se atribuyó a un crecimiento anormal de la bacteria *Aeromonas hydrophyla* y el conocido como mal de "la pata roja". Años más tarde, los ejemplares muertos que registramos en el Parque Nacional de Picos de Europa presentaban las mismas características que los que observamos en Pirineos, pero esta vez ya éramos capaces de identificar su agente causal: *Ranavirus* sp. (Price *et al.* 2014). Ahora sabemos que, contrariamente a lo que pensábamos hace sólo algunos años, *Ranavirus* es un patógeno multihospedador que no sólo produce mortalidad en poblaciones deprimidas, sino que es capaz de producir extinciones locales en poblaciones prístinas. Todo apunta a que *Ranavirus* está en proceso de expansión en Europa, una vez más, como consecuencia de la globalización, el comercio de especies y los movimientos incontrolados de animales, y su erradicación podría ser incluso más complicada que la de *Batrachochytrium* spp.

Actuaciones futuras

Actualmente son muchos los frentes abiertos en los que estamos trabajando para intentar frenar las enfermedades emergentes de los anfibios. Por supuesto, como en cualquier enfermedad infecciosa, la principal forma de actuar contra ella es evitar su propagación, por lo que nunca debemos bajar la guardia y seguir estrictamente los protocolos de desinfección del material de campo en nuestros trabajos, que básicamente consisten en sumergirlos en lejía, o en cualquier otro desinfectante usado en sanidad animal como Virkon, aclarar con agua limpia y dejar secar completamente antes de ser utilizados en otro lugar. También es fundamental divulgar el problema y concienciar a la sociedad en general sobre el riesgo de entrar en contacto estrecho con los animales y, sobre

todo, del enorme riesgo que supone mover ejemplares y materiales de un sitio a otro. Hay mucho trabajo por hacer con los diferentes usuarios de los cursos acuáticos, incluyendo pescadores, cazadores, herpetólogos, piragüistas y un largo etc, para conseguir que no realicen prácticas de riesgo que podrían ser catastróficas para los anfibios.

Además, hay que seguir luchando para que se implementen medidas legales para regular más severamente, e incluso prohibir, el comercio de anfibios. La reciente reincorporación de *Bd* en el listado de especies exóticas invasoras ha sido un éxito, pero de nada servirá si no estamos atentos para que se apliquen las leyes. Para el caso de *Bsal*, actualmente estamos trabajando para constituir un sistema de vigilancia temprana de la enfermedad a nivel europeo que podría evitar la llegada de este nuevo patógeno a la Península Ibérica.

Uno de los campos en los que estamos trabajando actualmente es el control de especies hospedadoras y reservorios. En Peñalara, la casi desaparición de *A. obstetricans* ha hecho que los niveles de infección bajen mucho, aunque, como vimos, la presencia de larvas invernantes de *S. salamandra* asegura la permanencia de *Bd* en el medio. Por eso, en los últimos años estamos realizando experimentos piloto en campo y laboratorio para comprobar si el control de este reservorio puede romper el ciclo anual de infección de nuevos ejemplares. En algunas lagunas y charcas permanentes, y con la ayuda de voluntarios, hemos retirado después del verano una enorme cantidad de larvas de *S. salamandra*, que han sido mantenidas en laboratorio hasta su metamorfosis. Con esta acción, en la primavera temprana, las nuevas larvas del año de *B. spinosus* y de *S. salamandra* no han entrado en contacto con larvas invernantes de *S. salamandra* altamente infectadas.

Otro campo abierto es el uso de bacterias anti-*Bd* para proporcionar una ayuda extra a los animales. Recientemente, hemos aislado una cepa de *Pseudomonas fluorescens* de ejemplares adultos de *A. obstetricans* de Peñalara que, en el laboratorio, inhibe el crecimiento de *Bd* (L.A. Davis *et al.* datos no publicados). Así, los ejemplares en fase larvaria de *A. obstetricans* que estamos reintroduciendo en Peñalara reciben antes un baño de estas bacterias (aunque con cautela dado que *Pseudomonas aeruginosa* y *P. flourescens* pueden comportarse como patógenos en herpetos inmunodeprimidos), y pronto sabremos si les están proporcionando una ayuda efectiva para controlar el hongo.

Figura 5. Instalaciones del Centro de Cría de Anfibios Amenazados de la Sierra de Guadarrama

Estos ejemplares liberados con las bacterias simbiontes anti-*Bd* han sido criados en el Centro de Cría de Anfibios Amenazados de la Sierra de Guadarrama, donde mantenemos una colonia cautiva desde 2008 creada a partir de los poquísimos ejemplares que conseguimos capturar tras el brote agudo de la enfermedad (Fig. 5). Desde entonces, hemos venido reforzando la población liberando cientos de metamórficos criados en cautividad que, de momento, nos ha permitido establecer dos nuevos núcleos de cría en el Parque y mantener viva la población. Además, con la creación del Parque Nacional de la Sierra de Guadarrama, hemos empezado a establecer nuevas poblaciones a cotas más bajas, pero dentro del Parque, y en áreas anteriormente ocupadas por la especie, donde hipotéticamente, las temperaturas más altas impedirán que *Bd* resulte letal para los animales.

Por supuesto, estos trabajos de reintroducción se han realizado tras el análisis genético de las poblaciones existentes (Albert *et al.* 2015), al igual que estamos haciendo en la actualidad en las zonas afectadas por *Ranavirus* para futuras reintroducciones. En este caso, y al contrario que contra *Bd*, nuestros primeros ensayos de inmunización de los animales son más esperanzadores, y pronto podremos probar en el campo si realmente son efectivos.

Tras casi dos décadas intentando combatir las nuevas enfermedades de los anfibios, es hora de encontrar soluciones. Aunque se trata de una tarea difícil de conseguir, debemos explorar cualquier posibilidad, por pequeña o descabellada que pueda parecer, con el convencimiento de que si no hacemos nada, nunca avanzaremos en el control de esta pandemia.

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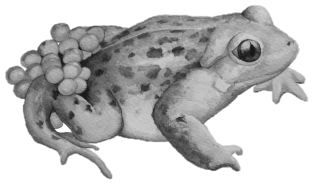

CAPÍTULO 2

PRIMERA CITA DEL HONGO QUITRIDIO *BATRACHOCHYTRIUM DENDROBATIDIS* EN EL NORTE DE ÁFRICA

FIRST RECORD OF THE CHYTRID FUNGUS BATRACHOCHYTRIUM DENDROBATIDIS IN NORTH AFRICA

PRIMERA CITA DEL HONGO QUITRIDIO *BATRACHOCHYTRIUM DENDROBATIDIS* EN EL NORTE DE ÁFRICA

RESUMEN

La quitridiomicosis, enfermedad producida por el hongo *Batrachochytrium dendrobatidis* (*Bd*), es una de las causas más importantes del declive de anfibios en todo el mundo. La rápida expansión de su distribución en cientos de especies de anfibios es verdaderamente alarmante. En España, se han detectado brotes de quitridiomicosis y mortalidades masivas asociadas en varias especies en diferentes zonas del país. Sin embargo, en Marruecos no se ha realizado un muestreo pormenorizado. El objetivo del presente estudio es llevar a cabo el primer muestreo de *Bd* en el norte de Marruecos para determinar la presencia, prevalencia e intensidad de la infección en las poblaciones de anfibios. Se han muestreado 203 especímenes de seis familias diferentes y nueve especies, capturados en 51 lugares distintos y la presencia de *Bd* se confirmó solamente en 4 anfibios de tres localidades: Disc*oglossus scovazzi* en Tetuoan; y *Hyla meridionalis* y *Pelobates varaldii* en la región de Larache-Lakser Lakbir con muy baja prevalencia (Prevalencia total: 6%) y relativamente baja intensidad. Con este estudio se confirma la presencia de *Bd* en Marruecos y como consecuencia en el norte de África. La distribución observada de *Bd,* avala la hipótesis de una posible diseminación del patógeno desde la Península Ibérica al norte de Marruecos, aunque para confirmar del todo esta hipótesis serían necesario estudios moleculares y un muestreo sistemático para poder determinar cual es la situación real en la zona.

FIRST RECORD OF THE CHYTRID FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS* IN NORTH AFRICA

ABSTRACT

An important driver of amphibian declines is chytridiomycosis, a disease caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*). The rapid and widespread distribution of *Bd* infection across hundreds of amphibian species is alarming. In Spain, outbreaks of chytridimycosis and mass mortalities have been reported in many species and in different regions of the country but no systematic surveillance for *Bd* has been undertaken across Morocco. The aim of the present study is to conduct the first survey for *Bd* in northern Morocco in order to determine the presence, prevalence and intensity of infection in the amphibian populations. We sampled 203 amphibian specimens representing six families and nine species captured from 51 sites and *Bd* was confirmed in just 4 amphibians collected from 3 sites: Disc*oglossus scovazzi* in the Tetouan region; and *Hyla meridionalis* and *Pelobates varaldii* in the Larache-Lakser Lakbir region with low prevalence (6%) and relatively low intensity. In our study, we confirm the presence of *Bd* in Morocco and consequently in North Africa. The observed distribution of *Bd* supports the hypothesis of a dissemination of *Bd* from Iberian Peninsula to the northern Morocco. However, confirmation of this hypothesis awaits molecular studies and a systematic survey of the area in order to determine the exact situation in the region.

INTRODUCTION

An important driver of amphibian declines is chytridiomycosis, a disease caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*; Berger *et al.* 1998, Daszak *et al.* 1999, 2003, Ron & Merino-Viteri 2000, Bosch *et al.* 2001, Collins & Storfer 2003, Ron *et al.* 2003, La Marca *et al.* 2005, Wake & Vredenburg 2008, Walker *et al.* 2010). *Bd* is now known to be an emerging pathogen that is rapidly expanding its global range (Fisher *et al.* 2009) and now has attained a global distribution on all continents that contain amphibians (www.bd-maps.net). Research by Weldon *et al.* (2004) on the potential origin of *Bd* suggests that the panzootic originated in South Africa and has perhaps been dispersed by international trade in amphibians, becoming established around the word (Rödder *et al.* 2009). To date, the rapid and widespread distribution of *Bd* infection across hundreds of amphibian species is alarming.

In Spain, outbreaks of chytridimycosis and mass mortalities have been reported in many species and in different regions of the country (Bosch *et al.* 2001, Bosch & Martínez-Solano 2006, Walker *et al.* 2008, 2010). In the south of Spain, *Bd* is widely distributed (www.bd-maps.net); Bosch *et al.*, unpublished data), suggesting that its occurrence in the north of Morocco is plausible. The two regions are spatially proximate with similar mediterranean ecologies, however are separated by the strait of Gibraltar. In addition, ecological niche modelling has shown that the environmental envelope in the north of Morocco is suitable for the establishment of *Bd* where susceptible amphibians occur (Ron 2005, Rödder *et al.* 2009). The legal and illegal increase in the transport of animals for pet trade (Fisher & Garner 2007), and the possibility that the fungus could be vectored into Morocco on the feathers of water birds, are potential modes of transmission between Spain and Morocco. However, no systematic surveillance for *Bd* has been undertaken across this region, where several endemic species occur with a high ecological value. The aim of the present study is to conduct the first survey for *Bd* in north Morocco in order to determine the presence, prevalence and intensity of infection in the amphibian populations that occur within region. These data will allow us to understand the distributional patterns of *Bd*, providing essential data for the effective management and control of this emergent pathogen.

METHODS AND MATERIALS

Surveys for *Bd* were conducted between October 2005 and April 2009. During this period, we prospected the north of Morocco, which is primarily a mountainous area (central-western Rif and north part of middle Atlas). Samples from the plain of Gharb and the Mammora regions were provided by P. de Pous (University of Applied Science van Hall-Larenstein, the Netherlands). The Rif region forms a mountainous barrier that is relatively low in elevation (less than 2500 m) with approximately half of the surface occurring above 500 m. It is an extension of the Baetic Cordillera, which includes the Sierra Nevada in the south of Spain. The area is a sub-wet zone with an average annual rainfall between 800 and 1400 mm (Bab Taza: 1361 mm/year), and probably reaches 2000 mm on the highest summits, and wetlands are common. In comparison, the coastal fringe is dry, where rainfall does not reach 500 mm (Oued Laou, 473 mm). Across the coast, the summer is moderate in temperature (Oued Laou, mean maximum temperature of the hottest month: 28.6°C), however weather stations located inland record much higher temperatures (Chefchaouen, mean maximum temperature of the hottest month: 33.8°C). In general, winter is the season when thermal contrasts are most variable, according to the altitude and of the distance of the sea. The coastal fringe has a mild winter (Oued Laou, mean minimum temperature of the coldest month: 7.2°C), with high-altitude areas recording harsher winters (Bab Taza, mean minimum temperature of the coldest month: 2.3°C). Separated from the Rif region by the corridor of Taza, the middle Atlas is the western most of three Atlas Mountains chains existing in Morocco. Its geomorphological structure is primarily constituted by limestone (with an altitude more than 3000 m) and volcanic plates. The western façade, with the oceanic influence, is the most humid part (approximately 1000 mm/year). More continental than Rif, the middle Atlas experiences harsh winters, with snow occurring above 2000 to 2500 m elevation. The Riffian and the middle Atlas forests are rich and are characterized by a gradient of elevation on which are overlaid continental, oceanic and mediterranean influences. The plain of Gharb and the forest of Mammora are located at the northwest of Morocco. The first is a basin, which is characterized by a climate of the mediterranean type with an oceanic influence. The east-west rainfall gradient varies between 450 and 530 mm, with 80% of the total rainfall occurring during the winter. In its south, the forest of Mammora is located in the semi arid bioclimatic zone. It is in extreme limit of the natural distribution surface of the mediterranean cork oak. This marginal geographical location makes this ecosystem particularly fragile. All of these

prospected ecosystems are indispensable for animal species, particularly amphibians, and are experiencing increasing deterioration under the effect of sustained anthropogenic pressures.

Amphibian specimens were collected opportunistically from 28 sites on the Rif region, 11 sites on the middle Atlas and 12 sites on the Gharb plain and Mammora forest (Table 1; Fig. 1). During nocturnal and occasional diurnal surveys, we extensively searched all water bodies that exist in the survey areas for amphibian larvae. We also searched for adults in the areas surrounding ponds. Immediately after capture, we took samples including toe clippings from adults and swabs from the mouthparts from tadpoles (MW100-100; Medical Wire & Equipment Co, Corsham, UK). Toe clippings were preserved in 70% ethanol for laboratory analysis. After sampling, animals were returned to their place of capture. The intensity of infection was subsequently assessed in the laboratory using the quantitative PCR (qPCR) protocol described by Boyle *et al*. (2004).

RESULTS

We sampled 203 amphibian specimens representing six families and nine species captured from 51 sites (Table 1). Of these, 96 specimens were collected in the Rif Mountains, 42 in the Middle Atlas and 64 in the Gharb plain. *Bd* was confirmed in just 4 amphibians collected from 3 sites (Table 1, Fig. 1); *Bd* was found in Disc*oglossus scovazzi* in the Tetouan region and in *Hyla meridionalis* and *Pelobates varaldii* in the Larache-Lakser Lakbir region (total prevalence 6%). Rare detection of the fungus occurred, with 1/1 in Agnane (intensity of infection: 29.9 genome equivalents, GE), 2/5 in Larach-Lakser lakbir (intensity of infection: 60.3 and 395.9 GE) and 1/10 in Larach (intensity of infection: 0.4 GE), demonstrating a low prevalence of infection for the fungus across these three sites.

Table 1. Localities in northern Morocco where amphibians were sampled for *Batrachochytrium dendrobatidis* (*Bd*).

Date	Coordinates (UTM format)	Species	LHS	N	IA	*Bd* IL
Nov 2006	Near Talambote					
	(30S 301325, 3901676)	Ds	A	3	0	-
Nov 2006	Near Talambote					
	(30S 296364, 3918812)	Am	PM	1	0	-
		Ds	A	1	0	-
Nov 2006	Remilate, Tanger					
	(30S 243011, 3968202)	Bm	A	1	0	-

Date	Coordinates (UTM format)	Species	LHS	N	IA	*Bd* IL
Nov 2006	Lahcen, between Tanger and Tetouan					
	(30S 267670, 3937240)	Sa	A	4	0	-
			PM	2	0	-
Nov 2006	Agnane, near Tetouan					
	(30S 283666, 3935092)	Ds	PM	1	1	10-50
Nov 2006	Koudiate Drup					
	(30S 267908, 3914926)	Hm	A	1	0	-
Nov 2006	Moulay Abdesalam					
	(30S 273019, 3911252)	Sa	A	5	0	-
		Ds	A	1	0	-
Nov 2006	Koudiate Sbaa					
	(30S 316198, 3877741)	Ds	A	4	0	-
			J	2	0	-
		Sa	A	5	0	-
Nov 2006	West Ketama					
	(30S 351185, 3867558)	Ds	A	1	0	-
Nov 2006	West Ketama					
	(30S 358428, 3859824)	Ds	A	1	0	-
Nov 2006	West Ketama					
	(30S 351309, 3867347)	Sa	A	1	0	-
Nov 2006	West Ketama					
	(30S 357507, 3860272)	Am	A	1	0	-
Nov 2006	Akchour, Talambote NP					
	(30S 301325, 3901676)	Ps	PM	12	0	-
Nov 2006	Akchour, Talambote NP					
	(30S 302760, 3903012)	Am	PM	2	0	-
Nov 2006	Jbel Moussa, Near Ceuta					
	(30S 283414, 3974434)	Ds	A	3	0	-
		Sa	A	1	0	-
Feb 2007	20k from Larache-Lakslakbir					
	(29S 76944, 3881906)	Ds	A	1	1	50-100
		Hm	A	4	1	100-500
Feb 2007	Between Zinate and Larache					
	(30S 278118, 3923873)	Bm	A	2	0	-
Feb 2007	Between Zinate and Larache					
	(30S 271385, 3918656)	Bm	A	2	0	-
Feb 2007	Between Zinate and Larache					
	(30S 265300, 3916882)	Bm	A	4	0	-
Feb 2007	Between Zinate and Larache					
	(30S 261159, 3912327)	Bm	A	1	0	-
Feb 2007	Mokrissat					
	(30S 284012, 3871774)	Am	PM	1	0	-
Feb 2007	Jebel Sunna, near Chefchaouén					
	(30S 284719, 3890398)	Sa	PM	1	0	-
Feb 2007	near Bab Bouidir					
	(30S 395460, 3770906)	Am	L	21	0	-
Aug 2007	Bab Bouidir					
	(30S 397165, 3770577)	Hm	A	1	0	-
Feb 2007	Tazekka NP					
	(30S 392482, 3769065)	Sa	A	1	0	-

Date	Coordinates (UTM format)	Species	LHS	N	IA	*Bd* IL
Aug 2007	Tazekka NP					
	(30S 394293, 3771325)	Ps	A	1	0	-
		Bb	A	1	0	-
Aug 2007	Tazekka NP					
	(30S 394480, 3772051)	Ps	A	1	0	-
			J	2	0	-
		Ds	J	1	0	-
Aug 2007	Tazekka NP					
	(30S 393337, 3771961)	Bb	A	1	0	-
Aug 2007	Tazekka NP					
	(30S 392652, 3774512)	Am	L	4	0	-
Aug 2007	Tazekka NP					
	(30S 390033, 3772458)	Sa	L	3	0	-
		Ds	J	1	0	-
Sep 2007	Between Tlet and Zerkat					
	(30S 366709, 3858627)	Ds	J	1	0	-
Sep 2007	Between BabTaza and Chefchaouén					
	(30S 323068, 3875857)	Ps	L	13	0	-
		Ds	J	1	0	-
Sep 2007	Near Talambote					
	(30S 302161, 3901983)	Am	L	1	0	-
Sep 2007	Way to Souk Khemis-des-Beni-Arouss					
	(30S 279104, 3905682)	Ds	J	10	0	-
Dec 2008	Between Khenifra and Itzer					
	(30S 274115, 3647848)	Bv	A	1	0	-
Dec 2008	Between Khenifra and Itzer					
	(30S 284069, 3642310)	Ds	J	2	0	-
Dec 2008	Between Khenifra and Itzer					
	(30S 284992, 3642900)	Ds	J	2	0	-
Jan 2009	South Sidi Yahya al Rharb					
	(29S 747855, 3788587)	Pv	L	1	0	-
Jan 2009	East Salé					
	(29S 712135, 3765885)	Pv	L	2	0	-
Jan 2009	East Salé					
	(29S 712108, 3767089)	Hm	L	1	0	-
		Pw	A	1	0	-
Feb 2009	South Oualidia					
	(29S 493990, 3607337)	Bb	L	1	0	-
Mar 2009	Mamora forest					
	(29S 721979, 3786336)	Pv	L	5	0	-
Mar 2009	Mamora forest					
	(29S 732621, 3796472)	Pv	L	5	0	-
Apr 2009	Mamora forest					
	(29S 732621, 3796472)	Pv	L	5	0	-
Apr 2009	Mamora forest					
	(29S 739204, 3771390)	Pv	L	5	0	-
Apr 2009	Larache					
	(29S 771002, 3881305)	Pv	L	10	1	0-10
Apr 2009	South Sidi Yahya du Rharb					
	(29S 753741, 3793514)	Pv	L	5	0	-

Date	Coordinates (UTM format)	Species	LHS	N	IA	*Bd* IL
Apr 2009	South Sidi Yahya du Rharb					
	(29S 745156, 3787886)	Pv	L	2	0	-
			Ps	A	3	0 -
Apr 2009	West Sidi Allal el Bahraoui					
	(29S 722308, 3768165)	Hm	L	4	0	-
		Pv	L	4	0	-
Apr 2009	East Kénitra					
	(29S 737246, 3785471)	Pv	L	5	0	-
Apr 2009	South Kénitra					
	(29S 718310, 3787768)	Pv	L	6	0	-
Apr 2009	South Taza					
	(30S 397532, 3772137)	Sa	A	1	0	-

Species: ***Am- Alytes maurus, Ds- Discoglossus scovazzi, Bm- Bufo mauritanicus, Sa- Salamandra algira, Hm, Hyla meridionalis, Ps- Pelophylax saharicus, Bb- Bufo bufo, Pb- Pseudepidalea boulengeri*** **(= North-African populations of *Bufo viridis* s.l.),** ***Pv- Pelobates varaldii, Pw- Pleurodeles waltl.*** **Life history stage (LHS): A- adult, L- larvae, J- juvenile, M- metamorph. N = sample size, IA = number of infected animals, range of Bd infection loads (Bd IL) in genomic equivalents detected using quantitative PCR.**

DISCUSSION

In Morocco, many populations of amphibian species are endangered and local declines of several species have been principally attributed to two threats: habitat destruction and pollution. However, no work has been conducted previously to assess the presence of *Bd* on the amphibians living in the country. In our study, we confirm the presence of *Bd* in Morocco and consequently in North Africa. This region of the world is not free of *Bd*, a finding that is in accordance with the models of Ron (2005) and Rödder *et al.* (2009), which predicted suitable habitats for *Bd* in north Morocco. The niche distribution models of Ron (2005) assigned a maximum overlap index of 1 to this region, which means that 10 of 10 models predicted the survival of *Bd*, should susceptible amphibian species exist.

The presence of *Bd* in Morocco and particularly in the north part of the country can be explained by several mechanisms. The presence of chytrid fungus in peninsular Iberia, especially in the south of Spain where it is broadly distributed, may have been vectored to the northern part of Morocco by anthropogenic or natural processes. For example, during a

survey in southern Spain performed in 2004, 31% of 86 collected samples and 29% of 17 sampled localities were *Bd*-positive (Bosch *et al.*, unpublished data). The border between the two countries experiences the extensive transport of industrial and agricultural products and animals across the strait of Gibraltar. Moreover, the presence of two Spanish enclaves at the north of Africa without customs and borders with the remaining of continental Spain makes the possible contamination of North Africa by *Bd* more real. However, natural vectors may also vector *Bd*. The fungus is able to survive and remain infectious for between 3 and 6 weeks in the environment in the absence of an amphibian host (Johnson and Speare 2003), may attach to plant debris and micro-organisms within sterile environments, and can perhaps persist in soil as do some other members of the phylum Chytridiomycota (Johnson and Speare 2005). These life-history characteristics of chytrid fungi allow prediction (and possible prevention) of its potential accidental transport via the movement of soil such as in extraction industries, in contaminated soil on vehicles or footwear of hikers or in plant nursery products (Laurance *et al.* 1997, Johnson *et al.* 2003, Johnson & Speare 2005). However, the dissemination by water birds through the migration routes between Europe and Africa via the Gibraltar strait may be responsible for facilitating the spread of the pathogen across this region, and this process is largely uncontrollable, rendering effective biosecurity of northern Africa unlikely.

Fig. 1. Map of northern Morocco showing *Batrachochytrium dendrobatidis* (*Bd*) sampling locations that were *Bd*-positive (closed triangle) and *Bd*-negative (open circles).

Our surveys were by no means exhaustive and further research on both the distribution and effect of the pathogen across this region is

needed. Our data shows: 1) three *Bd*-positive sites (1/1 in Agnane, 2/5 in Larach-Laks lakbir and 1/10 in Larach); and 2) a clustering of *Bd* in the western part of northern Morocco, just in the south of Tangitana peninsula (Fig. 1). This distribution supports the hypothesis of a dissemination of *Bd* from Iberian Peninsula to the northern Morocco. However, confirmation of this hypothesis waits molecular typing of isolates of *Bd* that have been recovered from these infected Moroccan populations.

Our results suggest a systematic survey of the area is warranted in order to determine the exact situation in the region. Furthermore, biosecurity and population management measures warrant consideration to prevent further amphibian species loss owing to this emergent pathogen.

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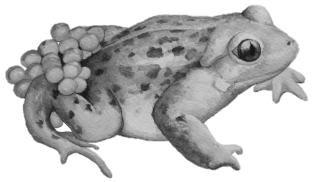

CAPÍTULO 3

EVIDENCIAS DE LA INTRODUCCIÓN DE LA QUITRIDIOMICOSIS Y DE LA AFECCIÓN LETAL AL SAPO PARTERO BÉTICO (*ALYTES DICKHILLENI*)

EVIDENCE FOR THE INTRODUCTION OF LETHAL CHYTRIDIOMYCOSIS AFFECTING WILD BETIC MIDWIFE TOADS (ALYTES DICKHILLENI)

EVIDENCIAS DE LA INTRODUCCIÓN DE LA QUITRIDIOMICOSIS Y DE LA AFECCIÓN LETAL AL SAPO PARTERO BÉTICO (*ALYTES DICKHILLENI*)

RESUMEN

Batrachochytrium dendrobatidis es un patógeno impredecible para las especies de anfibios europeas, cuya distribución y alcance están siendo probablemente subestimados con los estudios de campo existentes. Los episodios de mortalidades masivas registrados en Europa indican que dentro de las especies no estudiadas, las investigaciones deberían centrarse en especies de la familia *Alytidae*. Aquí, describimos los resultados combinados de muestreos de campo y observaciones de laboratorio de ejemplares de *Alytes dickhilleni* colectados en el campo. Nuestros datos sostienen la hipótesis de que *B. dendrobatidis* ha emergido recientemente en al menos dos localizaciones disjuntas del rango de distribución de la especie y muestran que no hay evidencia de infección por el patógeno en la mayoría de las poblaciones del rango de distribución de la especie. Las larvas trasladadas al laboratorio procedentes de lugares con presencia del patógeno sufrieron un 70% de mortalidad, al contrario de las que fueron recogidas de lugares no infectados y tanto la intensidad como la carga de infección fueron asociados con mortalidad en los animales colectados de lugares infectados. En respuesta a este estudio han surgido diversas iniciativas de conservación, incluyendo el establecimiento de una colonia cautiva de seguridad, una campaña de concienciación ciudadana y el desarrollo de pruebas experimentales de mitigación de la enfermedad.

EVIDENCE FOR THE INTRODUCTION OF LETHAL CHYTRIDIOMYCOSIS AFFECTING WILD BETIC MIDWIFE TOADS (*ALYTES DICKHILLENI*)

ABSTRACT

Batrachochytrium dendrobatidis is an unpredictable pathogen for European amphibian species, and existing field surveillance studies likely underestimate the scope of its distribution and effects. Mass mortality episodes recorded in Europe indicate that investigations of unstudied species should focus on members of the frog family Alytidae. Here, we report the combined results of a field survey and laboratory observations of field collected *Alytes dickhilleni*. Our data support the hypothesis that *B. dendrobatidis* has recently emerged in at least two disjunct locations in the species range and populations across much of the species range lack evidence of infection pathogen. Tadpoles taken into the laboratory from sites with infection experienced 70% mortality, unlike those taken into the laboratory from uninfected sites, and both infection and strength of infection was associated with mortality in animals collected from infected locations. Several conservation interventions are underway in response to our study, including the establishment of a captive assurance colony, a public awareness campaign, and experimental tests of disease mitigation schemes.

INTRODUCTION

The chytridiomycete fungus *Batrachochytrium dendrobatidis* is an extremely important pathogen of wildlife with respect to biodiversity conservation. The fungus is responsible for amphibian mass mortality, population declines, and possible species extinction at an intercontinental scale (Fisher *et al.* 2009). Disease emergences across the Neotropics and Australia are the worst on record and have caused catastrophic declines of numerous hosts in both regions (Berger *et al.* 1998, Lips *et al.* 2006, 2008, Skerratt *et al.* 2007, Fisher *et al.* 2009). In contrast, since its detection in Europe in the late 1990s, infection with *B. dendrobatidis* has been associated with mortality of a handful of European species in the wild; far fewer than are known to carry infections (Bosch *et al.* 2001, Bosch & Martínez-Solano 2006, Garner *et al.* 2006, Bovero *et al.* 2008, Walker *et al.* 2008, Garner *et al.* 2009a, Bielby *et al.* 2009, Ohst *et al.* 2011, Sztatecsny & Glaser 2011, Rosa *et al.* 2012). Published sampling for Europe is strongly spatially biased, includes only a subset of European amphibian species diversity (www.bd-maps.eu/) and many studies are extremely limited in scope and effort (Adams *et al.* 2008, Federici *et al.* 2008, Ficetola *et al.* 2011). This fact suggests that the documented list of European amphibians known to be infected with *B. dendrobatidis* and experiencing mortality is underestimated. Alternatively, some un- or under-sampled species may truly be resistant to infection (e.g., Bielby *et al.* 2008, Luquet *et al.* 2012), but this cannot be distinguished from lack of exposure to *B. dendrobatidis* without combined spatial surveillance and experimentation.

At the species level, the link between infection and disease is not straightforward (Garner *et al.* 2011, Luquet *et al.* 2012). Even when detectable mortality of a highly susceptible host species has been reported for multiple locations (Bosch *et al.* 2001, Walker *et al.* 2010, Pasmans *et al.* 2010, Rosa *et al.* 2012), the same species may not suffer from the lethal form of the disease at other sites where it is infected at high prevalence (Walker *et al.* 2010). Conversely, at locations where infection is detected but lethal disease has not been observed, cryptic mortality may occur (Tobler & Schmidt 2010). Studies restricted to a spatial investigation of infection therefore present uncertainties when attempting to assess the risk that infection presents to a host species. Combining spatial studies of prevalence of infection with in situ or ex situ experimental tests of virulence can potentially distinguish between tolerance of infection versus unobserved lethal chytridiomycosis. For obvious reasons, clarifying the

relationship between infection with *B. dendrobatidis* and amphibian mortality has greater conservation merit than simply describing parasite distribution (Garner *et al.* 2012).

Developing the database for resistance, tolerance, or susceptibility for all of Europe's amphibians is an enormous research task. The potential conservation threat chytridiomycosis may pose to the European amphibianfauna calls for a more rapid, but still reasoned approach to risk assessment. Previous mass mortalities episodes and population declines recorded in Europe showed that the family Alytidae is a strong candidate for detecting both infection and lethal disease, if they occur. Mortality in one member of the genus *Alytes* (*A. obstetricans*) is the flagship case of lethal chytridiomycosis in Europe (Bosch *et al.* 2001, 2007, Walker *et al.* 2010, Rosa *et al.* 2012). Infection and death has also been described for two other members of the family, *Alytes muletensis* (Walker *et al.* 2008) and *Discoglossus sardus* (Bielby *et al.* 2009). Other members of the genus *Alytes* exhibit traits (prolonged larval period and restricted species range) that should predispose them to declines due to chytridiomycosis (Martínez-Solano *et al.* 2004, Gonçalves *et al.* 2007, Bielby *et al.* 2008), which strongly argues for targeted studies of previously unsampled *Alytes* spp.

The betic midwife toad (*Alytes dickhilleni*) is a recently described species (Arntzen and García-Paris, 1995) that is the sister taxon to *A. muletensis* and possibly *Alytes maurus* (Martínez-Solano *et al.* 2004, Gonçalves *et al.* 2007). *A. dickhilleni* is distributed across an extremely restricted species range, occurring in mountains and the nearby plains of six provinces located in the southeast of Spain. It is listed as vulnerable by the IUCN, but no attempt has been made to ascertain if *B. dendrobatidis* is infecting this species or capable of killing post-metamorphic animals, as is true for both *A. obstetricans* and *A. muletensis* (Bosch *et al.* 2001, Walker *et al.* 2008, 2010). In this manuscript, we describe a two-phase study of the distribution of infection and lethal disease in *A. dickhilleni*. In the first phase, we surveyed for the presence of infection in wild populations of *A. dickhilleni*, a study that covered the entire species range. In the second, after determining where infection was located, we used an ex situ experimental approach (Tobler & Schmidt 2010) to determine if the presence of infection was associated with mortality due to chytridiomycosis in animals near to, or completing, metamorphosis.

METHODS AND MATERIALS

To ascertain the distribution and prevalence of infection in *A. dickhilleni* we first selected 30 sample sites covering the entire species range (Fig. 1). We focussed on sampling overwintered larvae because this life history stage has proven to be the most appropriate life history stage for detecting infection in two congeners (Walker *et al.* 2008, 2010). This was not possible at several sites, so young of the year tadpoles were sampled as an alternative (Table 1). To collect samples for molecular diagnosis of infection we swabbed the oral disc of each tadpole using a sterile swab (MW100, Medical Wire UK), a method proven to be reliable in previous studies of *Alytes* spp. tadpoles (Walker *et al.* 2008, 2010; Tobler and Schmidt 2010). At one location oral disks were excised from euthanized tadpoles and used for analysis. Our intention was to sample a mínimum of 20 tadpoles however this could not be achieved comprehensively due to poor tadpole abundance at some locations and an absence of tadpoles at others. At these latter sites we sampled smaller numbers of recently metamorphosed animals or adults by taking toe clips (Table 1).

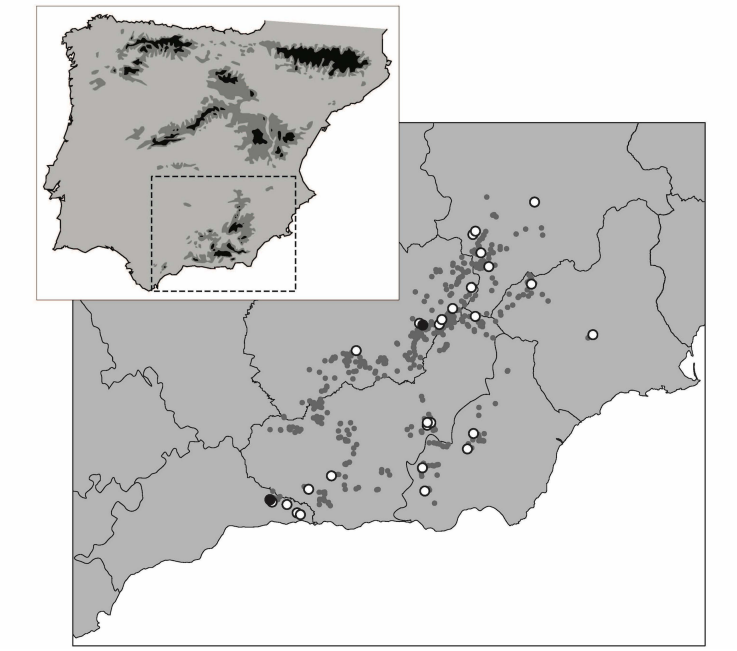

Figure 1. Sampling locations, including sites where *Batrachochytrium dendrobatidis* infection was detected (filled, black circles) and those where it was not (open circles). Small grey points show all known breeding sites for the species (from Bosch & González-Miras 2012).

In all cases recently metamorphosed animals were dead at time of sampling, and discovered that way. Swabs were refrigerated and stored dry

while toe clips were stored in ethanol until DNA extraction and qPCR amplification as per Boyle *et al.* (2004). Extractions were diluted 1:10 before real-time PCR amplification, performed in duplicate, with *B. dendrobatidis* genomic equivalent (GE) standards of 100, 10, 1, and 0.1 GE. When only one replicate from any sample amplified, we ran this sample a third time. If the third amplification did not result in an amplification profile, we considered the sample negative for infection. We used a Potthoff–Whittinghill's test of overdispersion to determine if risk of disease was homogeneous among sites (Potthoff and Whittinghill 1966). To do this, we condensed the UTM values for each sampled location into 10 x 10 km^2 cells. For the null hypothesis, we assumed that the number of infections per cell followed an expected Poisson distribution.

Table 1. Summary of sampling effort and qPCR analysis of field samples

Site	UTM x	UTM y	Alt (m)	Sample	LHS	Prev (99% CPCI)	GE mean	VMR
Albacete 1	547775	4264778	1321	20 swabs	YOY	0	-	-
Albacete 2	548263	4262150	1124	20 swabs	YOY	0	-	-
Albacete 3	548976	4262444	1118	3 swabs	YOY	0	-	-
Albacete 4	551157	4251097	1358	20 swabs	YOY	0	-	-
Albacete 5	554814	4242480	1154	20 swabs	YOY	0	-	-
Almeria 1	506909	4103323	2130	20 swabs	OW	0	-	-
Almeria 2	510777	4087945	1549	20 swabs	OW	0	-	-
Almeria 3	542640	4118482	1830	3 toe-clips	RM	0	-	-
Almeria 4	546738	4127735	1482	20 swabs	OW	0	-	-
Granada 1	436927	4087844	1316	17 swabs	NOW	0	-	-
Granada 2	450349	4100057	1377	20 swabs	OW	0	-	-
Granada 3	512655	4137144	2023	20 swabs	OW	0	-	-
Granada 4	513184	4135332	1995	10 swabs	OW	0	-	-
Granada 5	513522	4136045	2030	8 swabs	OW	0	-	-
Granada 6	547507	4210131	1482	3 toe clips	RM	0	-	-
Jaén 1	463077	4178001	1500	6 oral discs	YOY	0	-	-
Jaén 2	510055	4201811	1205	20 swabs	OW	0.95(0.68-1.00)	653.68	740.69
Jaén 3	510617	4200161	1257	20 swabs	NOW	0	-	-
Jaén 4	521022	4203158	1668	20 swabs	OW	0	-	-
Jaén 5	522555	4206471	1719	20 swabs	OW	0	-	-
Jaén 6	528958	4210948	1677	20 swabs	YOY	0	-	-
Jaén 7	540949	4224510	1600	9 toe clips	RM	0	-	-
Málaga 1	404929	404929	1080	20 swabs	OW	1(0.77-1.00)	100.00	27.41
Málaga 2	405361	4081292	701	11 swabs	NOW	0.36(0.07-0.77)	469.5	856.76
Málaga 3	414736	4079961	947	20 swabs	YOY	0	-	-
Málaga 4	420409	4072607	289	20 swabs	NOW	0	-	-
Málaga 5	421703	4071624	374	2 toe clips	A	0	-	-

Site	UTM x	UTM y	Alt (m)	Sample	LHS	Prev (99% CPCI)	GE mean	VMR
Murcia 1	583085	4278154	905	20 swabs	YOY	0	-	-
Murcia 2	586330	4228098	1216	20 swabs	YOY	0	-	-
Murcia 3	628110	4196409	900	19 swabs	YOY	0	-	-

Swabs of oral disks were collected from alive tadpoles at 25 sites, excised oral mouth disks from euthanized tadpoles were used at one site and toe clips were collected from dead metamorphosed animals at four sites.

We assessed the consequences of infection ex situ by broadly following the experimental protocol of Tobler and Schmidt (2010). Overwintered tadpoles were collected on the third week of September of 2009 from three locations, one where we had detected infected tadpoles (La Rahige in Sierra Tejeda), and two where we had not detected any evidence of infection (two sites located in Cazorla, Segura y Las Villas Natural Park). To minimize any possible effects of time in captivity, we restricted the collection of experimental tadpoles to 1 day per location. Consequences of this collection approach included uneven sample sizes among groups in the laboratory observations (Sierra Tejeda, n = 16; 2 sites in Cazorla, Segura y Las Villas Natural Park, n = 19 or 10).

We transported tadpoles to Bioparc Fuengirola where they were housed individually in plastic cups containing 1 l of aged tap water. Tadpoles were fed every two days and water was changed at each feed. Temperature in the experimental unit was kept at a constant 18°C and on a 12:12-h light schedule. Tadpoles were maintained in this manner until 14 days post-metamorphosis or death. Metamorphosis was defined as the day the tail was first observed to be resorbed (dark tail stub present, but stub not protruding beyond the "heels" of the hind limbs). To determine infection status, one hind toe was clipped from each animal either 7 days after metamorphosis or, in the event the animal died before 7 days post-metamorphosis, on the day of death. Toe clips were subjected to the same laboratory procedure, as were field surveillance samples. Animals that survived for 14 days post-metamorphosis were treated with Itraconazole (Itrafungol, Esteve) following Garner *et al.* (2009b) and returned to their site of collection after two months' quarantine and no evidence of infection was detected using molecular diagnostic techniques applied after quarantine. We used a Cox proportional hazards (CPH) model to determine whether time to metamorphosis, infection with *Bd* as a categorical value (infected yes/no) or mean GE were significant predictors of survival of tadpoles collected from the field. Because infection data were

unavailable for one of the Sierra Tejeda animals that died on day 42, it was excluded from the CPH model.

RESULTS

We sampled a mean of 14.4 individuals (min. 3, max. 20) at 30 breeding sites distributed across the six provinces where *A. dickhilleni* is endemic (Fig. 1; Table 1). The risk of infection was not homogeneously distributed across sites (Potthoff–Whittinghill's test of overdispersion; T = 11004.33, p = 0.001) and infection was detected at three sites, clustered in two 10x10 grid squares (Fig. 1). The grid square containing two of the sites where infection was detected includes the recreational area of La Rahige located in the Sierra Tejeda, Málaga. One of these two sites is a completely artificial breeding site (cattle tank, La Rábita Fountain, site Málaga 1, Table 1), while the other is a manmade pool (La Rahige, site Málaga 2, Table 1) located in small, slow-moving, naturally occurring and permanent stream within the recreational area. Stream flow downstream from La Rahige has been partially dammed to increase water depth in the pool for recreational purposes. The third positive location is an undisturbed stream (Guadahornillos, site Jaén 2, Table 1) located near to the Roblehondo Biological Station CSIC in Cazorla, Segura y Las Villas Natural Park, located in another grid cell several cells away from the cell containing La Rábita Fountain and La Rahige.

Prevalence at the two Sierra Tejeda sites varied: tadpoles at La Rábita Fountain were 100% infected, while at La Rahige only 36% of tadpoles were infected. In comparison, tadpoles at Guadahornillos were more similar to La Rábita Fountain in terms of prevalence (95%). Burden of infection was, on average, high at all three locations and mean GE was similar amongst sites (Table 1). All recently metamorphosed juveniles and the two adults that were tissue sampled also tested negative for infection.

Survival of post-metamorphic *A. dickhilleni* in captivity was not consistent across treatments. Experimental tadpoles from the Sierra Tejeda were the only animals that tested positive for infection at time of death or seven days postmetamorphosis and experienced substantial mortality. Tadpoles collected from the sites where infections were not detected during the field survey experienced significantly less mortality when compared to the Sierra Tejeda group (Fig. 2). After excluding the one animal for which we lacked infection data, animals that died in the Sierra Tejeda group (12/15) were almost all infected (10/12) and the majority died within 60

days after the start of the laboratory observations (n = 10, minimum number of days to death = 6, mean number of days to death ± SD = 24.6 ± 15.4, mean GE ± SD = 12,741.4 ± 10,734.8, range GE = 0–29,367.6). The two other tadpoles from Sierra Tejeda that died did so 143 and 261 days after the start of the laboratory observations and exhibited infectious burdens of, respectively, 0.1 and 3.9 mean GE. Cox proportional hazards analysis revealed that infection status (yes/no, p = 0.002) but not mean GE (p = 0.470) nor time to metamorphosis (p = 0.329) significantly increased probability of death. Being infected increased the relative instantaneous mortality hazard by a factor of 15.7 times.

DISCUSSION

The spatial pattern and number of infected populations we detected strongly suggests that *B. dendrobatidis* is a recently emerged parasite of *A. dickhilleni*. There are few robust spatial studies of *B. dendrobatidis* distribution available for European hosts, but of those that are published the majority involve congeners of our study species, and report a significantly greater proportion of sites with infections than we found (Walker *et al.* 2008, 20% sites with infected animals; Walker *et al.* 2010, 25% sites with infected animals; Tobler *et al.* 2012, 61.5% sites with infected animals; this study, 10% of sites with infected animals). Walker *et al.* (2008, 2010) identified other Iberian locations where recent introductions of *B. dendrobatidis* into *Alytes* spp. populations had occurred, including human-mediated introduction into populations of *A. muletensis* in the 1990s with little or no evidence of post-introduction dispersal (Walker *et al.* 2008). The pattern described by our study fits this latter case: two highly distinct foci of infection where prevalence is at or near to saturation in overwintered tadpoles (La Rábita Fountain and Guadahornillos), indicative of two recent introductions, and limited evidence of fungal dispersal to nearby sites (La Rahige: located near to a saturated population, but with significantly lower prevalence). Both of our proposed sites of introduction are locations where human activity that could promote pathogen introduction is common. Guadahornillos is located just 1.5 km away from an important location for biological research and Sierra Tejeda Natural Park is a multi-use recreational park frequently visited by amateur herpetologists looking for the species at its *terra typica*.

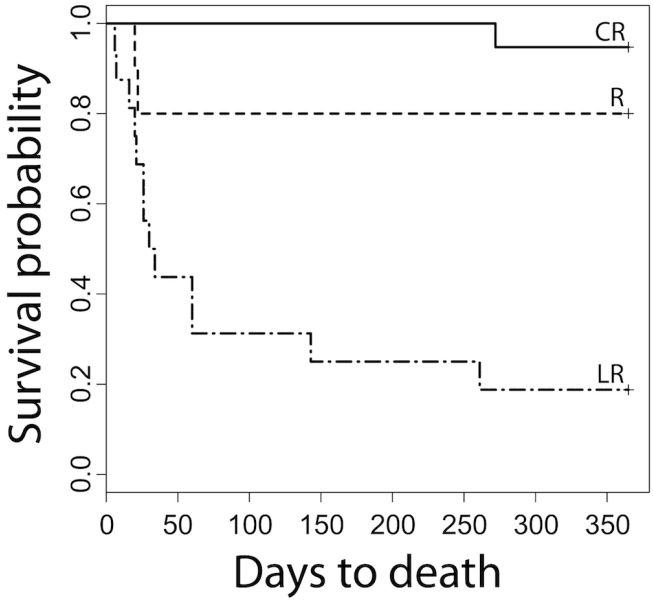

Figure 2. Survival curves for tadpoles sampled at three locations, two within Cazorla Segura y Las Villas Natural Park (CR, R) and at Sierra Tejeda (LR).

Our spatial study revealed no observable evidence of disease-linked mortality in recently metamorphosed juveniles. Mass mortality at metamorphosis is the signature of lethal chytridiomycosis in *A. obstetricans* (Bosch *et al.* 2001, Bosch *et al.* 2007, Walker *et al.* 2010) and some field mortality has been observed in *A. muletensis* (Walker *et al.* 2008, JB personal observations), but we never encountered large numbers of dead animals of any life history stage at any location. The few recently metamorphosed and dead animals that we did encounter in nature did not test positive for infection (Table 1). The results of laboratory observations of field collected animals, however, showed that it is highly probable that *A. dickhilleni* metamorphs at Sierra Tejeda are experiencing substantial, cryptic mortality as nearly 70% of overwintered tadpoles collected from this area died in captivity. Mortality was significantly associated with infection status, typically occurred soon after the onset of the laboratory observations and was commonly associated with heavy burdens of infection.

These results also provide the first experimental evidence that infected tadpoles are capable of maintaining infections over long periods of time without external forcing of infection (Briggs *et al.* 2010) and dying as a result. This finding is important because increased exposure to *B. dendrobatidis* through forcing of infection can be strongly influenced by transmission from other infected hosts, and has been linked to increasing risk of mortality through the accumulation of fungal load (Briggs *et al.*

2010, Vredenberg *et al.* 2010) but the relationship between host density and transmission is unclear (Rachowicz & Briggs 2007). Because our experimental animals were housed individually we can exclude transmission among hosts as an influence, removing the effect of persistent external forcing from the individual disease dynamic. Most of the Sierra Tejeda tadpoles spent more than 20 days without being exposed to any transmission vector, two greater than 140 days, and still died after metamorphosis. Infections acquired earlier during development were therefore sufficient to elicit death long after the initial transmission event, as was the case for *A. obstetricans* tadpoles (Tobler & Schmidt 2010). This may be an indication that costs accrued by tadpoles during this time relate to post metamorphic death, which has been seen in species with relatively short larval periods (Garner *et al.* 2009, Luquet *et al.* 2012). It seems sensible, therefore, that a more prolonged larval period with an associated prolonged period of sustained infection should be costly to the infected host.

Studies of chytridiomycosis in *Alytes* spp. do not always provide a definitive link between mortality caused by chytridiomycosis and species decline. In Peñalara Natural Park, *A. obstetricans* was driven locally extinct due to disease emergence (Bosch *et al.* 2001, Martínez-Solano *et al.* 2003, Bosch & Rincón 2008). A recent publication by Tobler *et al.* (2012) outlines the alternative and describes a lack of a clear relationship between the presence of infection and host population dynamics although the authors do postulate that their system may have suffered historical declines due to disease. We lack quantitative data on population responses at locations where *A. dickhilleni* is infected. Irrespective of the inconsistencies, evidence for introduction of *B. dendrobatidis* into populations of a high-risk species coupled with evidence for substantial mortality due to disease is cause for conservation intervention. Bioparc Fuengirola and its Foundation, in association with the Amphibian Ark, have established a biosecure facility for the maintenance of a disease-free captive assurance colony of *A. dickhilleni*. This is coupled with more general conservation activities including habitat restoration and improvement, local education programs and other efforts to raise awareness regarding amphibian decline, conservation and chytridiomycosis. Disease monitoring and the development of methods to mitigate infection in the wild are underway, as part of the in situ, 'Betic Midwife Toad Conservation Project' lead by Bioparc Fuengirola and in collaboration with the National Museum of Natural Sciences of Madrid (CSIC).

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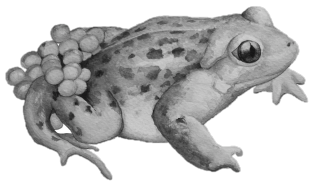

CAPÍTULO 4

EVALUACIÓN DE LA PRESENCIA DE QUITRIDIOMICOSIS EN EL TRITÓN DEL MONTSENY (*CALOTRITON ARNOLDI*), CRÍTICAMENTE AMENZADO, EN EL NORDESTE ESPAÑOL

CHYTRIDIOMYCOSIS SURVEILLANCE IN THE CRITICALLY ENDANGERED MONTSENY BROOK NEWT, CALOTRITON ARNOLDI, NORTHEASTERN SPAIN

EVALUACIÓN DE LA PRESENCIA DE QUITRIDIOMICOSIS EN EL TRITÓN DEL MONTSENY (*CALOTRITON ARNOLDI*), CRÍTICAMENTE AMENZADO, EN EL NORDESTE ESPAÑOL

RESUMEN

La quitridiomicosis, una enfermedad causada por el hongo patógeno *Batrachochytrium dendrobatidis* (*Bd*), ha causado severos declives en las poblaciones de anfibios de diferentes áreas de España. El tritón del Montseny (*Calotriton arnoldi*) es endémico de la región montañosa del Montseny en Cataluña, noreste de España. Como parte de su plan de conservación es necesario evaluar con especial atención el estado de salud de sus poblaciones, que aún es incierto. De 2007 a 2011, llevamos a cabo muestreos en el Parque Natural del Montseny y analizamos la presencia de *Bd* en 158 tritones (*C. arnoldi*), 14 salamandras (*Salamandra salamandra*) y 2 sapos comunes (*Bufo spinosus*) mediante PCR cuantitativa a tiempo real (qPCR). Todas las muestras resultaron negativas para la presencia del patógeno, lo que sugiere que *Bd* está ausente en la región o presente en un nivel tan bajo que no se ha podido detectar. La implementación de sistemas de vigilancia de enfermedades en la fauna silvestre, especialmente en especies amenazadas es crucial para la detección de infecciones subclínicas y la pronta adopción de medidas paliativas.

CHYTRIDIOMYCOSIS SURVEILLANCE IN THE CRITICALLY ENDANGERED MONTSENY BROOK NEWT, *CALOTRITON ARNOLDI,* NORTHEASTERN SPAIN

ABSTRACT

Chytridiomycosis, a disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) has caused significant declines of amphibian populations in different areas of Spain. The critically endangered Montseny Brook Newt (*Calotriton arnoldi*) is endemic to the mountain region of Montseny in Catalonia, Northeast Spain. As part of its conservation plan special attention was needed to evaluate the population health status, which remained uncertain. From 2007 to 2011, we conducted a survey in Montseny Natural Park and examined 158 Montseny brook newts, 14 fire salamanders and 2 common toads for the presence of *Bd* using quantitative real-time Taqman PCR (qPCR) assay. All samples were negative to this pathogen suggesting that *Bd* is absent in the region or present in such a low level that it was undetected. The implementation of disease surveillance in wildlife, especially in endangered species, is of crucial importance for the detection of subclinical infection and prompt adoption of counter measures.

INTRODUCTION

The Montseny brook newt (*Calotriton arnoldi*) is an endemic amphibian only found in seven mountain streams of Montseny Natural Park (Catalonia, Northeast Spain). Based on its extremely small distribution range (40km^2) and population size (1500 adults) (Amat 2004, Amat & Carranza 2005, Amat & Carranza 2007) is considered critically endangered by IUCN (Carranza & Martínez-Solano 2009).

Chytridiomycosis, a disease caused by the waterborne chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), has been associated with amphibian mortality and population declines on at least four continents (Berger *et al.* 1998, Bosch *et al.* 2001, Fisher *et al.* 2009). In Spain *Bd* has been vastly studied in certain areas, especially in Peñalara National Park (Bosch *et al.* 2001, Bosch and Martínez-Solano 2006) and Ibon Acherito (Walker *et al.* 2010) were it caused severe declines in common midwife toads (*Alytes obstetricans*). Moreover it has been detected in reintroduced captive bred populations of the vulnerable Mallorcan midwife toad (*Alytes muletensis*) (Walker *et al.* 2008) and in many amphibian species in Doñana National Park (Hidalgo-Vila *et al.* 2012). Thus, *Bd* could be considered a potential risk for the conservation of Montseny brook newt, and still this endemic species has not been screened for infection. The aim of this study was to assess for the presence and prevalence of *Bd* in this critically endangered species.

METHODS AND MATERIALS

Fieldwork was conducted between October 2007 and November 2011 and we obtained samples from 158 animals. The population is split in two differentiated and non-connected sectors in the Tordera river basin, eastern and western (Montori & Campeny 1991, Carranza & Amat 2005, Valbuena-Ureña 2010). Fifty-four samples came from the three populations of the eastern sector (refereed in the Table 1 as A1, A2, A3) and 104 samples came from the four populations of the western sector (refereed in the table as B1, B2, B3, B4). Sample size was calculated using Win Episcope 2. 0 (Clive, Edinburgh, UK) and with a hypothetic infection prevalence of 2% and a population of 1500 animals we aimed for at least 142 samples to achieve a 95% confidence level. We considered that an ideal ecosystem approach to disease monitoring would also have to include other amphibian species with wider ranges. Thus we decided to include samples from 14 adult fire salamanders (*Salamandra salamandra*) and two adult

common toads (*Bufo spinosus*), which were captured in the mountain streams of Montseny. We sampled each animal by firmly running a cotton tipped swab (MW100 swabs, Medical Wire & Equipment) over the skin of ventral surface including undersides of thighs and toes. All animals were handled with gloved hands and all equipment was disinfected using 1% Virkon between sites. All swab-samples were stored dry and refrigerated until analysed.

DNA swab extraction was made using PrepMan Ultra (Applied Biosytems) (Hyatt *et al.* 2007). The amount of DNA present in each sample was calculated using real time PCR, with a *Bd*-specific Taqman Assay (Boyle *et al.* 2004) in a CFX96 Real Time PCR Detection Systems (Bio-Rad) and using standards containing known genome-equivalents. Each sample was run per duplicate and infection load was measured as the number of zoospore genome equivalents per swab. Individuals were considered *Bd* positives when the results of the two replicates were consistent and obtained zoospore equivalents that were >0.1. We used an internal positive control (IPC) to measure PCR inhibition in randomly selected samples that tested negative for *Bd* infection. Following the methodology of Hyatt *et al.* (2007), a VICTM labelled synthetic amplicon was used as the IPC (VICTM dye, Applied Biosystems). The IPC was included in one of each duplicate well as 1 µl 10x Exo IPC mix and 0.5 µl 50x Exo IPC DNA.

RESULTS

None of the samples collected from wild amphibians showed evidence of amplification by the *Bd*-diagnostic primers. IPCs showed that there was no evidence for PCR inhibition in any sample. All results are summarized in Table 1 and data has been deposited in the EU *Bd*-surveillance archive at www.bd-maps.eu.

DISCUSSION

This study demonstrates for the first time that *Bd* is not present in Montseny brook newts, or in other amphibian species of Montseny Natural Park. Thus our results indicate that this fungus is not an actual risk for the Montseny brook newt population at present time. Some aspects of the biology of the species may be protective against invasion and spread of an introduced pathogen. The population density is very low and very limited dispersal has been recorded by capture-recapture study (Amat, unpublished

data). Moreover, all developmental life stages are fully aquatic making movements between streams impossible by natural means. On the other hand aquatic amphibians with limited distribution are in general recognised as the most endangered by extinction due to chytridiomycosis (Bielby *et al.* 2008), therefore the risk should not be underestimated.

Table 1. Field-testing for *Batrachochytrium dendrobatidis* in Montseny Natural Park between 2007 and 2011 with negative PCR results

Year Month	Population Code	*C. arnoldi*	*S. salamandra*	*B. bufo*
2007-October	A1	10		
	B1	4		
	B2	4		
2009-June	A1	13		
	B2	5		
2009-October	B1	2		
	B2	12		
	B3&B4	7		
2009-November	B4	9		
2010-April	A1	2		
	A3	4	1	
	B1	6	2	
	B3	14	4	
	B4	4		
2010-May	A1	1		
	A2	2		
	A3	1		
	B1	8		1
	B3	6		
2010-June	A2	3		
2011-March	B1	3		
	B2	8		
2011-May	A1	5		
	A3	3		
	B1	5		
	B2	4		1
	B3	2		
2011-Octuber	B2		4	
	B3	1	3	
2011-November	A1	10		
TOTAL		158	14	2

Populations A1, A2, A3 belong to the Eastern sector and B1, B2, B3 and B4 belong to the Western sector of Montseny Brook newts.

Surveillance for novel infectious disease surveillance is of paramount importance in the management of amphibian populations (Pessier & Mendelson 2010) given the disease-related aspects of global amphibian

declines. Despite the secretive behaviour of the Montseny brook newts we were able to collect samples from approximate 10% of the expected wild adult population. Our surveillance did not detect the presence of *Bd* so this could mean that the pathogen is absent in the region or that it is present in such low level that it was undetected. Assuming a disease low prevalence of 2% might have existed, there was a 96,57 % probability that we would have detected at least one positive individual by sampling 158 animals as calculated using Win Episcope 2.0 (Clive, Edinburgh, UK). It is also remarkable because we obtained samples in different months to minimize the possibility of seasonal variation in detection, as it is known that chytridiomycosis prevalence can vary dramatically depending on air temperature (Kriger & Hero 2007).

The impact of chytridiomycosis on representatives of the order Caudata is still poorly understood and variable symptomatology has been reported. This ranges from the loss of digits and skin discoloration in Sardinian newt (Bovero *et al.* 2008) to massive die-offs in salamander (Bosch & Martínez-Solano 2006). Ohst *et al.* 2011, reported that the alpine newt (*Ichthyosaura alpestris*) had one of the highest *Bd* prevalences (14,9%), after conducting an extensive surveillance in Germany that included many endemic amphibian species (urodele and caudate). Similarly, in Austria and in Spain (Doñana National Park) several species of caudate amphibians tested positive to *Bd* with variable prevalences (Hidalgo-Vila *et al.* 2012, Sztatecsny & Glaser 2011). All these data support the importance of including caudate amphibians for *Bd* surveillance. In future, surveillance for *Bd* will continue in Montseny Natural Park aiming for an early detection of infection in the Montseny brook newt and other more common amphibians. Management strategies are being implemented to avoid the introduction and dissemination of the pathogen by rangers and other occasional visitors, with specific formation about working routines and disinfection of materials.

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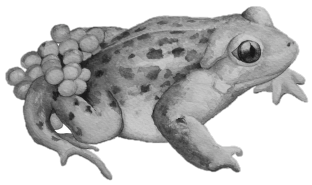

CAPÍTULO 5

LA TEMPERATURA MÍNIMA DEL AGUA DETERMINA A CORTO PLAZO LOS NIVELES DE INFECCIÓN POR EL HONGO QUITRIDIO DE LOS ANFIBIOS EN LARVAS DE *ALYTES OBSTETRICANS*

SHORT-TERM MINIMUM WATER TEMPERATURES DETERMINE LEVELS OF INFECTION BY THE AMPHIBIAN CHYTRID FUNGUS IN ALYTES OBSTETRICANS TADPOLES

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RESUMEN

Los anfibios son uno de los grupos de fauna silvestre más seriamente amenazados por las enfermedades infecciosas emergentes. En particular, la quitridiomicosis, causada por el hongo acuático *Batrachochytrium dendrobatidis*, es responsable de declives de anfibios a escala mundial. Los efectos que siguen a la introducción del patógeno a nivel poblacional dependen del contexto y están regulados por una larga lista de variables bióticas y abióticas. En particular, algunos estudios han demostrado que la temperatura tiene un papel clave en determinar las dinámicas de la infección debido a la naturaleza ectotérmica de los anfibios hospedadores y a que la tasa de crecimiento del patógeno también depende de la temperatura. Para evaluar la dependencia de la estacionalidad de la temperatura en las cargas de infección de una especie susceptible, seguimos las larvas de una población de zonas bajas de sapo partero común (*Alytes obstetricans*), en el centro de España, a lo largo de todo un año. Encontramos que la infección es altamente estacional y está inversamente correlacionada con la temperatura del agua, con las cargas más altas de infección durante los meses más fríos. Los impactos a corto plazo de la temperatura del agua, cuando las mínimas temperaturas se daban antes del muestreo son un mejor predictor de la carga de infección que los cambios de temperatura a largo plazo. Nuestros resultados son muy útiles para elegir el momento óptimo para la realización de muestreos y para determinar los momentos clave para realizar actuaciones de mitigación

SHORT-TERM MINIMUM WATER TEMPERATURES DETERMINE LEVELS OF INFECTION BY THE AMPHIBIAN CHYTRID FUNGUS IN *ALYTES OBSTETRICANS* TADPOLES

ABSTRACT

Amphibians are one of the groups of wildlife most seriously threatened by emerging infectious disease. In particular, chytridiomycosis, caused by the aquatic fungus *Batrachochytrium dendrobatidis*, is responsible for amphibian species declines on a worldwide scale. Population-level outcomes following the introduction of the pathogen are context dependent and mediated by a large suite of abiotic and biotic variables. In particular, studies have shown that temperature has a key role in determining infection dynamics owing to the ectothermic nature of the amphibian host and temperature-dependency of pathogen growth rates. To assess the temperature-dependent seasonality of infectious burdens in a susceptible host species, we monitored lowland populations of larval midwife toads, *Alytes obstetricians*, in Central Spain throughout the year. We found that infections were highly seasonal, and inversely correlated against water temperature, with the highest burdens of infection seen during the colder months. Short-term impacts of water-temperature were found, with the minimum temperatures occurring before sampling being more highly predictive of infectious burdens than were longer-term spans of temperature. Our results will be useful for selecting the optimal time for disease surveys and, more broadly, for determining the key periods to undertake disease mitigation.

INTRODUCTION

Spatiotemporal variation in the amount of clinical disease caused by pathogens is common in nature (Bjørnstad *et al.* 2002, Hosseini *et al.* 2004, Lofgren *et al.* 2007, Duncan *et al.* 2011). In many settings, this variation follows a marked seasonal pattern that is related to the thermal requirements of the pathogens (Waller *et al.* 2004, Cattadori *et al.* 2005) or to changes in the host density and its immune system (Laakkonen *et al.* 1999, Altizer *et al.* 2006, White *et al.* 2005, Cheng *et al.* 2009) Seasonality determines much of the external environmental variation influencing diseases, and several studies have shown that seasonality plays an important role in population ecology of both host and pathogen (Altizer *et al.* 2004, Tamerius & Comrie 2011). Therefore, by studying seasonal environmental variability it is possible to make predictions about changes in infection dynamics over time.

Chytridiomycosis is an amphibian specific disease that has been recognized as one of the main causes of recent amphibian declines and extinctions worldwide (Stuart *et al.* 2004, Olson *et al.* 2013). The causal agent, *Batrachochytrium dendrobatidis* (*Bd*) is a pathogenic fungus whose impacts on amphibian populations show strong spatial and temporal variation. Spatial variation in the prevalence of infection is highly heterogeneous and caused by taxonomic (under- and over-infected families), environmental (e.g., temperature range and precipitation at a site), and community-level (e.g., species richness) determinants (Olson *et al.* 2013). It is well known that some populations of susceptible species do not develop any symptoms of disease, even when many individuals are highly parasitised by *Bd* (Tobler *et al.* 2012), whereas others suffer severe declines or extinctions. This variation in the virulence of *Bd* is known to be caused by a complex interaction between biotic (host community structure, predatory microbiota, probiotic skin bacteria) and abiotic (altitude, temperature, UVB, climatic seasonality) factors (Briggs *et al.* 2007, Kriger & Hero 2007, Harris *et al.* 2009, Doddington *et al.* 2013, Schmeller *et al.* 2014). In temperate zones, with greater seasonality, the outcome of infection is usually more variable than in tropical areas (Savage *et al.* 2011), being more harmful in cool and high altitude regions (Bosch *et al.* 2001, Rachowicz *et al.* 2006, Walker *et al.* 2010). Within a region, seasonal environmental fluctuations have important consequences for the development of disease.

Among the many environmental factors that regulate populations of saprobic aquatic fungi, temperature has profound impacts on their

population dynamics and explains why many chytridiomycetes bloom with seasonal temperature changes (Sparrow 1968). *Bd in vitro* grows most rapidly between 17°C and 25°C. At temperatures above 28°C and below 10°C the growth rate decreases (Longcore *et al.* 1999, Piotrowski *et al.* 2004) and the pathogen is killed within short periods of time at temperatures of 37°C or above (Johnson *et al.* 2003). An increasing number of studies have revealed a strong influence of temperature on patterns of *Bd* infection in wild populations (Kriger & Hero 2007, Drew *et al.* 2006, Bosch *et al.* 2007, Knapp *et al.* 2011). Australian adult frogs show peaks of prevalence in the coolest months of the year (Kriger & Hero 2007, Retallick *et al.* 2004, Kriger & Hero 2006, Phillott *et al.* 2013) and the same pattern is known to occur in North America and Puerto Rico, where the epidemiology of *Bd* is strongly dictated by seasonality, transitioning from complete disappearance during summer to causing die-offs during winter (Ouellet *et al.* 2005, Andre *et al.* 2008, Bradley *et al.* 2010, Longo *et al.* 2010, Voordouw *et al.* 2010, Savage *et al.* 2011).

While *Bd* attacks the keratinized mouthparts of amphibian larvae, tadpoles survive as reservoirs without suffering obvious symptoms of disease (Briggs *et al.* 2005, Bosch *et al.* 2001). However, when metamorphosis occurs, the fungus invades the recently keratinized skin of the juvenile animal causing hyperkeratosis, impairment of osmoregulatory processes and triggering death in vulnerable species (Voyles *et al.* 2009). Mortality is dependent on an individuals intensity of infection, which also shows temporal variation depending on the year, breeding behaviour of the animals, density of the host or their body size and sex (Rowley & Alford 2007, Murray *et al.* 2013). Therefore, linking changing patterns of infection intensity to biotic and abiotic factors is important for understanding the rate of clinical disease that ultimately impacts host populations. In this study we aimed to assess the temperature-dependent seasonality of infectious burdens in tadpoles of a susceptible species in order to improve our ability to model and predict the impacts of chytridiomycosis across host populations.

METHODS AND MATERIALS

The study area is located in the agropastoral valley of the Duoro River in the municipality of Toro (Province of Zamora in Castilla-León, Spain). Fresh water springs are common and many flow into artificial troughs that are used by cattle. *Aytes obstetricans* (the common midwife toad)

breeds in these troughs, where the species reproduces twice a year (spring and autumn) and autumn tadpoles often overwinter.

Six troughs located between 693 and 772 m of altitude were selected for the study (Nueva de Bardales, UTM coordinates 30T 304137, 4588429; Valdespino, UTM coordinates 30T 300479, 4587707; Villares, UTM coordinates 30T 299974, 4586932; Perros, UTM coordinates 30T 297429, 4585493; Picarico, UTM coordinates 30T 295107, 4586056 and Marlota, UTM coordinates 30T 294955, 4583532). Selected troughs were similar in size and hold similar tadpole densities to those found across the Iberian Peninsula. Submerged dataloggers (HOBO Pro v2 Water Temperature Logger U22– 001, Onset Inc., Bourne, Massachusetts) in each trough provided a continuous half-hourly measurement of water temperature across one year, from February of 2010 to January of 2011. These sites were not privately owned or protected and therefore no permissions to access the troughs are required. On the other hand, because the studied species is protected by national legislation, animal care as well field permits to conduct this research were obtained from the Consejería de Medio Ambiente, Junta de Castilla y León, Spain (permit EP/CyL/20/2010).

Each month, we estimated the total number of *A. obstetricans* tadpoles present in every trough, as well the proportion of tadpoles below Gosner stage 36 (no, or rudimentary, hind limbs present)(Gosner 1960). Twenty tadpoles were captured at random with a small hand-net and swabbed gently with a sterile cotton swab (MW 100– 100, Medical Wire & Equipment) 20 times across their mouthparts. After swabbing, the tadpoles were released back into their troughs. According to the current national legislation no approval from an Animal Care and Use Committee is required for these procedures. DNA extractions from the swabs were performed using PrepMan Ultra (Applied biosystems) and the amount of *Bd* DNA present in each sample was measured through a CFX96TM Real-Time PCR Detection System (BIORAD) with a *Bd* -specific Taqman Assay (Boyle 2004). Each 96-well assay plate included a negative control and four different standards containing DNA from 100, 10, 1 and 0.1 *Bd* genome equivalents. We used an isolate from infected *A. obstetricans* in Northwest Spain (IA042, Ibón Acherito, Spanish Pyrennees) as a source of standards. Both, the standard isolate and the strain present at the studied area genetically closely related, being members of the *Bd* GPL lineage (unpublished results).

Infection load was measured as the number of zoospore equivalents per swab. Each simple was performed in duplicate and individuals were considered *Bd*- positive when the results of the two replicates were consistent and > 0.1 zoospore genome equivalents. If not, the simple was re-running and considered positive only if another positive result occurred. Prevalence was calculated as the percentage of infected individuals.

General Linear Mixed Models were applied to analyse variation in population averaged *Bd* loads (log transformed; x' = ln [x+1]) throughout eleven months (from March to January). Population averaged *Bd* loads were used instead of individual values, as our sampling unit was the population at each " site". Site was considered as a random factor, and average minimum and maximum daily water temperatures, tadpole abundance and tadpole development as covariates. These analyses were carried out using estimates of average minimum and maximum temperatures across six different periods of time (2, 5, 10, 15, 22 and 30 days prior to tadpole sampling). As February was the first month with temperature records we could not include this month in data analyses due to the lack of temperature data for January 2010. The mean square (MS) and the degrees of freedom (df) of the error terms were estimated following Satterthwaite' s method; this finds the linear combinations of sources of random variation that serve as appropriate error terms for testing the significance of the respective effect of interest. We chose this design, instead of random intercept + random slope models due to (a) the lack of significance of interaction terms with the random factor and (b) the analytical power. As the interaction terms [site x minimum temperature], [site x maximum temperature], [site x tadpole abundance] and [site x tadpoles development] were not significant, there is no need to develop a random intercept + random slope model. Further, random intercept + random slope models demand large sample sizes in order to attain high levels of statistical power; this is not our case, as we only have six different sites sampled in eleven months.

Standardized regression coefficients (β) were obtained for covariates as a measure of the sign and magnitude effects of predictor variables (*i.e.* analyses were carried out with standardized variables, such that their averages are zero and variances are one). For the random factor "site" we estimated the proportion of variance accounted for in each model. Homoscedasticity and normality of residuals of the General Linear Mixed Models were checked and did not deviate from the canonical assumptions.

Seven alternative models were compared with Akaike's second-order AIC corrected for small sample sizes (AIC_c) (Burnham & Anderson 2006) to assess their weights of evidence. All these models included the site, tadpole abundance and tadpole development as predictors, but varied according to the inclusion of temperature covariates. Six *a priori* models included the average minimum and maximum temperatures estimated across six different periods of time; 2, 5, 10, 15, 22 and 30 days before tadpole sampling. The seventh model did not include any temperature measurement, to control against the influence of temperature in determining the population averaged *Bd* loads of tadpoles (see Table 1). The strength of evidence of models was obtained using weights (W_i) derived from AIC_c figures, and their quotients were used to compare pairs of models. Parameter estimates (β, proportion of variance accounted for by "site" and R^2) were averaged using model weights (W_i). Such a model-averaged estimator compares favorably in terms of bias and precision against a single estimator extracted from the single best model. Finally, all possible subsets of the predictors using General Linear Mixed Models were estimated, considering each time span (2, 5, 10, 15, 22 and 30 days prior to tadpole sampling) and always including the random factor "site" (76 models; to control for the fact that our true sample unit was the population at each " site").

Table 1. Alternative models for *Batrachochytrium dendrobatidis* infection loads (in logarithm) in six different sites.

	AIC_c	W_i	W_t / W	Model R^2	Min T	MaxT	Abund	Develop	%Var sites
with temperature (2 days)	181.82	0.444	264284	0.523	-0.780	0.082	-0.013	-0.037	0.053
with temperature (5 days)	182.08	0.389	231832	0.522	-0.622	-0.079	-0.009	-0.039	0.052
with temperature (10 days)	184.25	0.131	78112	0.506	-0.392	-0.289	-0.024	-0.029	0.050
with temperature (15 days)	187.54	0.025	15085	0.480	-0.281	-0.378	-0.024	-0.024	0.050
with temperature (22 days)	189.85	0.008	4766	0.462	-0.106	-0.529	-0.029	-0.006	0.049
with temperature (30 days)	192.50	0.002	1268	0.440	-0.067	-0.546	-0.024	0.020	0.048
without temp	206.79	0.000		0.242			-0.451	0.087	0.038
weighted averages				0.519	-0.648	-0.047	-0.013	-0.036	0.052

The first six models include the average minimum and maximum water temperature (T) in six different time spans prior to tadpole sampling (*i.e.*, two, five, ten, 15, 22 and 30 days), taking into account other three predictor terms: tadpole abundance (abund), tadpole development (develop) and site. Sample

size is 66 (six sites x 11 months). AIC_c: AIC corrected for small sample sizes. W_i: model weights. W_t / W: quotient of strength of evidence dividing the weight (W_t) each model containing both maximum and minimum temperatures with the model without temperatures (W). Figures below Min T, Max T, tadpole abundance and development are standardized regression coefficients (β) obtained in mixed general linear models (β values inform about the magnitude and sign of the partial relationships of the predictor variables). Weighted averages: multimodel inference of standardized β regression coefficients considering the model weights W_i. The AIC_c figure for the null model (*i.e.*, not including any effect) is 208.04.

The influence of phenology on the population averaged *Bd* loads in tadpoles was tested by means of a three-order polynomial for month (March-1, January-11) using the residuals of the seven *a priori* models in Table 1. These post-hoc models attempt to quantify the magnitude of variance in population averaged *Bd* loads that occur across the year, but are not related to temporal changes in temperature, tadpole abundance and tadpole development.

All the statistical analyses were carried out using STATISTICA 10 (StatSoft Inc, Tulsa, Oklahoma)

RESULTS

Seasonal changes in water temperature were very consistent across study sites (see Fig. 1), with lower temperatures from January to March (around 8°C), and higher temperatures in late summer (August and September; around 20°C). The lowest minimum water temperature registered was 1.2°C (March), while the highest recorded maximum water temperature was 25.4°C (September). Maximum differences among sites in average monthly temperatures were around 4°C, ranging from 2.0°C in April to 5.4°C in August; these differences among sites were very similar considering both the monthly minimum and maximum temperatures. Diurnal temperature variations were on average 2.2°C across sites and throughout the study period, with highest recorded figures in June (3.5°C in average) and lowest figures in December and January (1.0°C).

For all sites, the prevalence of *Bd* infection in tadpoles was highly variable, with an average per site ranging from 5 to 100% (Fig. 1). Prevalence was strongly correlated with population averaged *Bd* loads, demonstrating a steep increase from zero to 100 zoospore equivalents, and

a nearly saturated prevalence above 200 zoospore equivalents. The relationship between these two variables approached linearity when prevalence was arcsin-transformed and zoospore load was log-transformed. Thus, 54.4% of variation in *Bd* prevalence across sites and throughout months was explained by a model including *Bd* load and site ($F_{6, 59}$ = 11.76, p<< 0.001); population averaged *Bd* load accounted for 49.6% of variance in *Bd* prevalence ($F_{1, 5}$ = 31.47, p = 0.002) while site accounted only for 2.7% of variance ($F_{5, 59}$ = 0.70, p = 0.626). For this reason, subsequent data analyses have been restricted to population averaged *Bd* load for the sake of brevity, considering the tight relationship between *Bd* prevalence and load, the very high prevalences measured, and according to the fact that prevalence did not reliably inform about the high variation in *Bd* load with prevalences greater than 75%.

Subsequent addition of minimum and maximum temperatures to models including tadpole abundance, tadpole development and site considerably increased the proportion of variance explained by population averaged *Bd* load, and the strength of evidence of models (Table 1). The model lacking temperature had a strength of evidence many times (> 1,000) lower than the models including average minimum and maximum temperatures 2, 5, 10, 15, 22 and 30 days before tadpole sampling. Moreover, models including both temperatures five or two days before sampling occurred had strengths of evidence > 200,000 times higher than models not including temperature.

The effect of temperature in explaining population averaged *Bd* load diminished from the short-term (*i.e.*, two days before tadpole sampling) through to mid-term (*i.e.*, 30 days before sampling), shown by the increase in AIC_c values and the decrease in model weights in Table 1. According to the quotient of model weights, considering average temperatures during the two days prior to tadpole sampling provided a model with a strength of evidence 222 times higher than the model that included average temperatures during the preceding 30 days.

Both minimum and maximum temperatures had a negative influence on population averaged *Bd* load, with the effect of minimum temperature (β = -0.648) being higher than that of maximum temperature (β = -0.047; see standardized regression coefficients and their weighted averages in Table 1). Thus, population averaged *Bd* load decreased across sites and months as temperature increased. Local tadpole abundance and development had a negligible influence on population averaged *Bd* load.

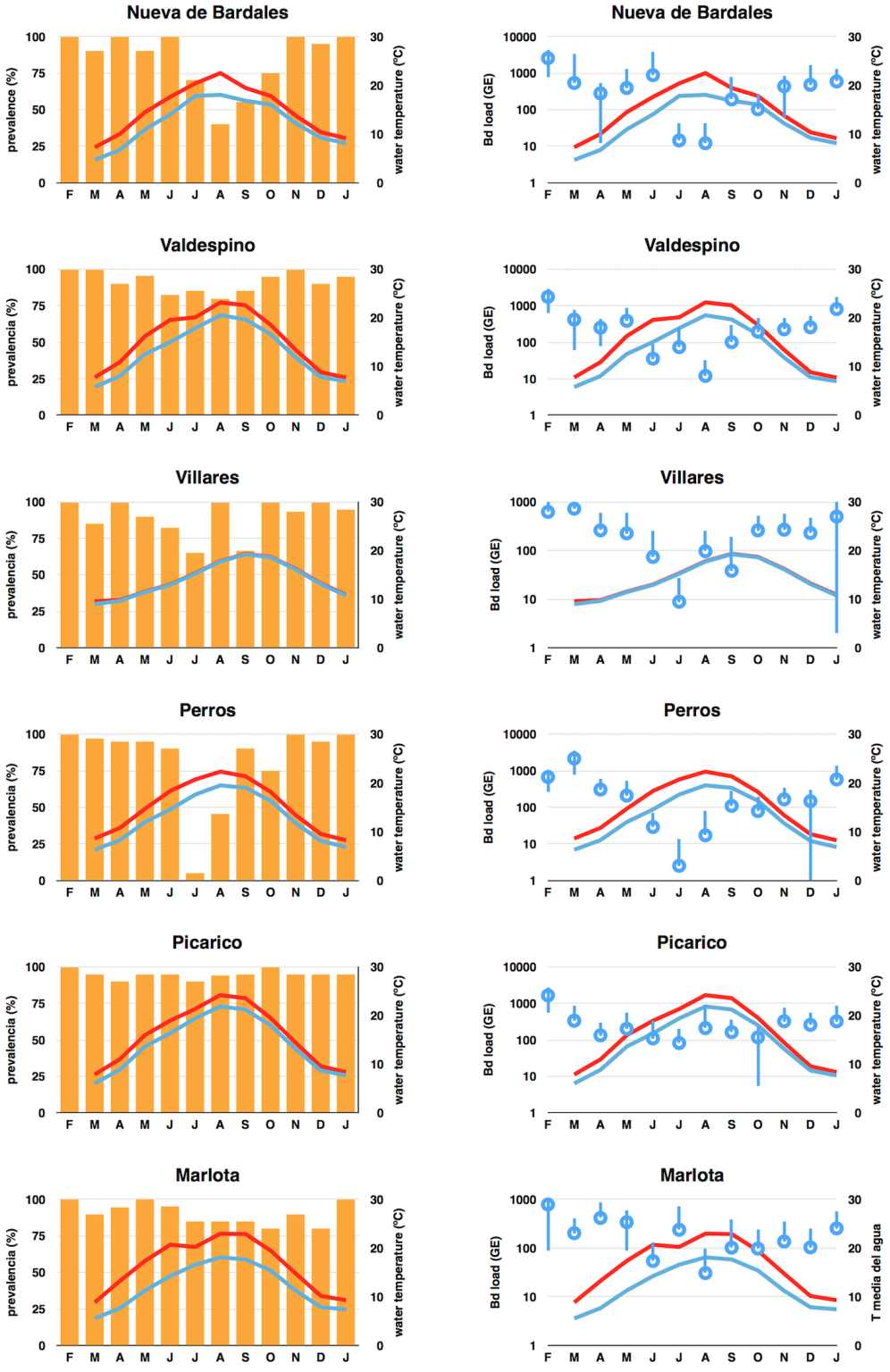

Figure 1. Prevalence and infection load of *Batrachochytrium dendrobatidis*. Prevalence (left) and infection load (right; in logarithm scale, GE, mean +/- SE) for populations of *Alytes obstetricans* tadpoles in six different sites throughout 12 months in each site. Monthly average minimum and maximum water temperature are shown in blue and red respectively.

The negative influence of average minimum temperature on population averaged *Bd* load steadily decreased from very short time spans of two days to longer periods of 30 days (see Fig. 2 for its influence in the time span of two days before tadpole sampling). The converse pattern was observed for the average maximum temperature, whose importance was higher averaging data for 22 and 30 days prior to tadpole sampling (see also β coefficients in Table 1).

The most parsimonious model, of all those possible, in explaining population averaged *Bd* load was one that included the average minimum temperature two days before tadpole sampling and the factor "site" (AIC_c = 173.7; R^2 = 0.522; $F_{6, 59}$ = 10.73, p<< 0.001), with a strong negative effect of minimum temperature (β = -0.696; $F_{1, 5}$ = 25.46, p< 0.001) and "site" explaining a low amount of variance (5.1%; $F_{5, 59}$ = 1.60, p = 0.174). A similarly parsimonious model (AIC_c = 173.9; R^2 = 0.520; $F_{6, 59}$ = 10.65, p<< 0.001) included minimum temperature five days before tadpole sampling (β = -0.694) and site as predictors (5.1% of variance). The first model, including the average maximum temperature during 30 days before tadpole sampling, had a considerably lower strength of evidence (AIC_c = 184.2; R^2 = 0.439; $F_{6, 59}$ = 7.69, p<< 0.001), with temperature having a strong negative effect (β = -0.631; $F_{1, 5}$ = 22.39, p< 0.001) and "site" explaining 4.9% of variance ($F_{5, 59}$ = 1.19, p = 0.326).

The proportion of variability in population averaged *Bd* load not accounted by the six a priori models in Table 1 including temperature (*i.e.*, model residuals) was explained to a very low extent by sampling date (using a cubic polynomial of month: 0.8– 2.2%; p > 0.71 in the six models).

In summary, temperature had an important role in determining the intensity of infections by *Bd*. Its effect was considerably more important when the average minimum temperature 2-5 days before tadpole sampling was taken into account, and the influence of average mínimum temperature on population averaged *Bd* load was considerably higher than that recorded for the average maximum temperature. Variability in population averaged *Bd* load that could be attributed to different localities was low, accounting for only 5% of the total variability. The influence of tadpole abundance and tadpole development on population averaged *Bd* loads was nearly negligible and the proportion of variability in population averaged *Bd* loads that was accounted by sampling date was very low.

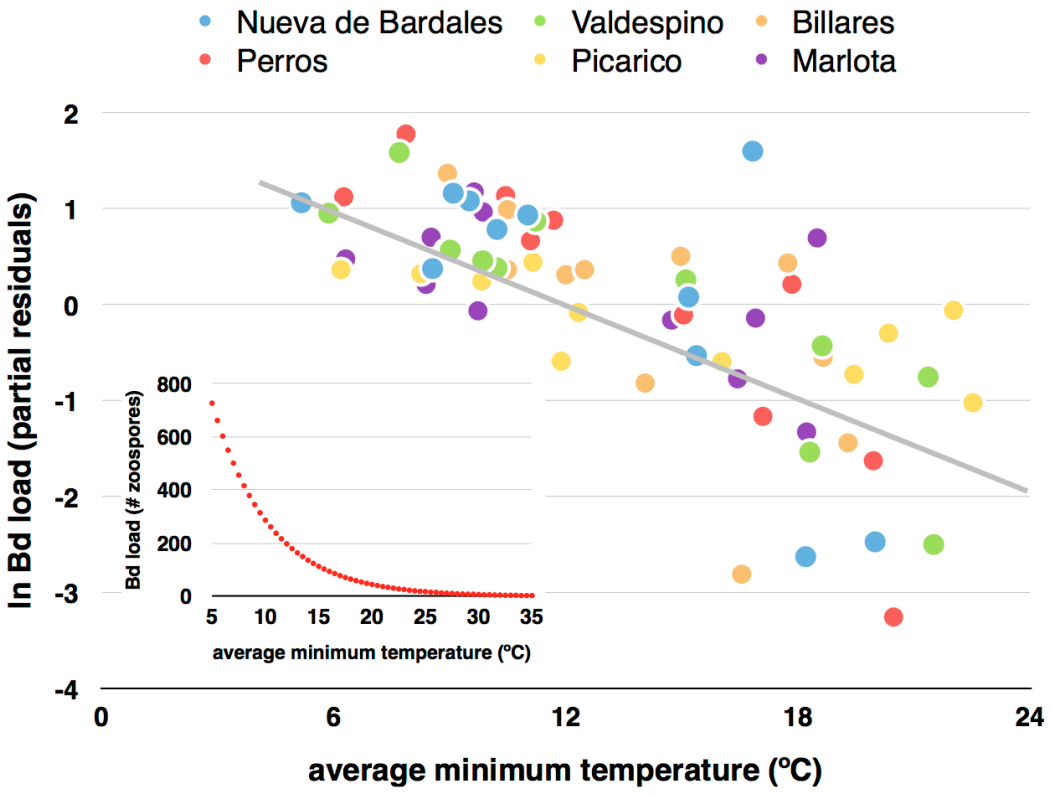

Figure 2. Average minimum water temperature and infection load of *Batrachochytrium dendrobatidis*. Partial residual plot illustrating the influence of average minimum water temperature two days before tadpole sampling, on *Batrachochytrium dendrobatidis* infection load (in logarithm) of *Alytes obstetricans* tadpoles from six different sites. Sample size is 11 months for each site. The residual plot shows the relationship between minimum temperature and *Bd* load given that the other two independent variables are also in the model (see with temperature-2 days in Table 1), therefore, partialling out their effects. The inner panel shows the modeled relationship between *Bd* load (average number of zoospores per tadpole) and average mínimum temperature two days before sampling.

DISCUSSION

Previous studies of *Bd*-infected *Alytes muletensis* tadpoles on the island of Mallorca showed that the survival of infected populations was site specific, and that this owed to the role of temperature in regulating the host/pathogen dynamic (Doddington *et al.* 2013). In our current study, we demonstrate across a replicated set of mainland *A. obstetricans* breeding sites that temperature exerts a similarly profound effect. This leads to pronounced seasonal cyclicity in *Bd* loads, with the highest burdens of

infection seen during the colder months. In other ecosystems various factors such as habitat type, density of the host, life history traits and virulence of the infecting strain of *Bd* have also been argued as possible causes of this variation in host susceptibility (Woodhams *et al.* 2008, Fisher *et al.* 2009, Olson *et al.* 2013).

Local weather conditions and their seasonal variation are known to have a large influence on pathogen-host dynamics (Fisher *et al.* 2009, Longo *et al.* 2010, Savage *et al.* 2011, Murray *et al.* 2013,). As seasonality is typically stronger in temperate climates, where numerous and serious cases of amphibian mass mortalities due to chytridiomycosis have been recorded, some authors have suggested that temperate zones are exposed to a higher risk of outbreaks of chytridiomycosis (Hof *et al.* 2011). This argument has some validity as temparate populations of *Rana muscosa* and *R. sierra*, which like *A. obstetricans* are highly vulnerable to chytridiomycosis, show high mortality at high-altitudes (Briggs *et al.* 2010, Knapp *et al.* 2011). However, in contrast to our findings, these studies have shown no effect of seasonality on the intensity of *Bd* infections. This likely owes to the fact that in these montane areas of the USA researchers cannot simple amphibians in the winter months due to the presence of the snow cover; our sample sites fail to freeze in winter and we are able to sample throughout the year.

We witnessed increasing infection loads while temperature decreased, *i.e.* peaks of infection in the winter. Many other authors have obtained similar results in the laboratory (Berger *et al.* 2004, Andre *et al.* 2008, Bustamante *et al.* 2010, Murphy *et al.* 2011) and also in the field, however studies have most often been undertaken in tropical areas, or have not covered the entire seasonal variation in temperature (Retallick *et al.* 2004, Kriger *et al.* 2007, Bradley *et al.* 2010, Savage *et al.* 2011, Knapp *et al.* 2011, Forrest and Schlaepfer 2011). Our study corroborates these results in a temperate climate, by monitoring the infection on tadpoles monthly during a year in the field. The higher importance of minimum over maximum water temperature is easily understandable considering that maximum temperatures allow for the availability of lower temperatures throughout the day that are more favorable for the chytrid fungus.

We found that the effect of average minimum temperature was higher than that recorded for the maximum temperature during the same period of time. Although the range of averaged maximum temperatures recorded during summer months (15.3 - 23.6°C) was within the optimal range of *Bd* growth (17 - 25°C) (Piotrowski *et al.* 2004), we observed the

lowest infection loads across this period. We have previously used mathematical models and analysis of the temperature-dependent expression of *Bd*-infected adult *Silurana tropicalis* immune-related genes to show that temperature, zoospore growth rates and immune-related clearance all interact to determine *Bd* loads (Ribas *et al.* 2009). While our current study is in focused on larval, rather than adult stages, it is likely that a similar interaction between host and pathogen life-history variables are interacting with temperature to determine the intensity of infections. During spring and autumn we saw similar ranges of averaged maximum temperatures (9.8 - 20.7°C and 8.9 - 19.5°C, respectively) and also similar infection intensity between these two seasons. While the range of temperatures during winter (7.3 - 12.6°C) was outside of the optimal range for *Bd*, we saw the highest infection loads precisely in this season. This suggests a failure in the ability of the tadpoles to clear infection, rather than the effect of the pathogens growth alone. The increased susceptibility that we see is likely related to the poorer functioning of the immune system at temperatures below 10°C, a fact that has been strongly supported in a study of temperature-dependent immune inhibition in *S. tropicalis* (Ribas *et al.* 2009). However, far less is known about the temperature-dependency of immune function in larval amphibians and here we can only speculate that our findings may owe to temperature-dependent immune-inhibition that may impact the adaptive and innate arms of the immune system including tadpole ability to synthesise antimicrobial peptides. That we see the greatest impact of temperature just before sampling episodes shows that these temperature-related impacts on susceptibility are dynamic. However, further work is required to disentangle the causal relationships between rates of *Bd* growth and tadpole immune responses.

Partialling out the effects of the other variables, average minimum water temperature two days before sampling shows a strong negative relationship with the logarithm of average population *Bd* load (see the inner panel in Fig. 2). The lower 95% confidence interval of the modeled relationship makes the infection equal to zero zoospores when the average minimum temperature is 25°C (a very similar relationship and temperature cut-off point is obtained analyzing the time span of 5-days before tadpole sampling). Nevertheless, the minimum water temperature predicted for completely eliminating *Bd* infection at the population level was 35°C. Therefore, if minimum water temperature is higher than 25°C during two-five days before, there is a very high probability that most, but not all, tadpoles lose the infection. This result matches those previously found in *A. obstetricans* in Switzerland (Geiger *et al.* 2011), and confirm the valid cut-

off point of ca. 30°C to completely eliminate *Bd* infection in the wild considering that above this temperature *Bd* in culture begins to die (Rachowicz *et al.* 2006, Forrest & Schlaepfer 2011).

We found that tadpole abundance and tadpole development exert a negligible effect on *Bd* infection and, in agreement with our findings, detailed studies of *R. muscosa* tadpoles (Knapp & Morgan 2006) also failed to find a significant relationship between *Bd* presence and larval stage. On the other hand, some laboratory and field studies (Rachowicz & Briggs 2007) indicate a clear influence of the density of infected individuals in the rates of *Bd* transmission. Despite the fact that some of these studies considered *Bd* prevalence while others *Bd* load, these contrasting results could be related to differences among species in their intrinsic susceptibility to infection by *Bd* and requires further study.

Determining the relationship between environmental variables across local scales and their relationship to pathogen growth, disease infection levels, and ultimately episodes of mass mortality, will allow us to improve our ability to model and predict the impacts of this infection across host populations. This will be useful, for example, in refining survey strategies by selecting the periods with the highest burdens of infection and, therefore, reducing sample size required without losing statistical power. Additionally, and more importantly, this knowledge will improve our ability to make evidence based management decisions to undertake disease mitigation attempts during the periods with the lowest burdens of infection. Ultimately, this will enhance the decision making process with regard to conservation measures that could enhance the survival of endangered or threatened species.

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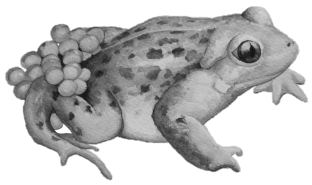

CAPÍTULO 6

MANEJO GENÉTICO DE UNA POBLACIÓN DE ANFIBIOS TRAS UN BROTE DE QUITRIDIOMICOSIS

GENETIC MANAGEMENT OF AN AMPHIBIAN POPULATION AFTER A CHYTRIDIOMYCOSIS OUTBREAK

MANEJO GENÉTICO DE UNA POBLACIÓN DE ANFIBIOS TRAS UN BROTE DE QUITRIDIOMICOSIS

RESUMEN

Una epidemia de quitridiomicosis, enfermedad provocada por el hongo patógeno *Batrachochytrium dendrobatidis*, indujo un declive masivo de las poblaciones del sapo partero común (*Alytes obstetricans*) en el macizo de Peñalara (Parque Nacional Sierra de Guadarrama, centro de España) durante los años 1997-2001. El brote de la enfermedad causó la desaparición de aproximadamente el 90% de las poblaciones, dejando sólo 8 núcleos reproductores. En respuesta a este declive poblacional, se puso en marcha un programa de cría en cautividad en 2008. Las poblaciones fueron mantenidas por separado para minimizar una posible depresión por exogamia. Aquí, examinamos índices de diversidad genética y la estructura poblacional en las poblaciones remanentes para aportar información que pueda ser útil para futuras reintroducciones. Los análisis de 10 loci microsatélites mostraron una fuerte estructura genética entre los diferentes núcleos reproductores, lo que sugiere que existe un escaso intercambio genético y una relativamente baja diversidad genética. En concordancia con el cuello de botella demográfico observado en los últimos años, encontramos evidencias de una fuerte reducción de la diversidad genética. Nuestros resultados sugieren que el programa de cría en cautividad debería mezclar animales de los diferentes núcleos reproductores dentro del rango de la Sierra de Guadarrama, pero evitando utilizar ejemplares del núcleo poblacional más divergente genéticamente.

GENETIC MANAGEMENT OF AN AMPHIBIAN POPULATION AFTER A CHYTRIDIOMYCOSIS OUTBREAK

ABSTRACT

An epidemic of the disease chytridiomycosis, caused by the pathogenic fungus *Batrachochytrium dendrobatidis*, induced a massive decline of populations of the common midwife toad (*Alytes obstetricans*) inhabiting the Peñalara Massif (Guadarrama National Park, Central Spain) in the years 1997–2001. The disease outbreak caused the disappearance of about 90 % of populations, leaving only eight remnant breeding populations. In response to the disease-induced population decline, a captive breeding program was started in 2008. Populations were kept separate to minimize possible outbreeding depression. Here, we examined indices of genetic diversity and population structure in these remnant populations to inform future reintroductions. Analysis of ten microsatellite loci showed strong genetic structure between breeding sites suggesting little genetic exchange and relatively low global genetic diversity. In accordance with the demographic bottleneck observed in the last years we found strong evidence for a reduction in genetic diversity. Our results suggest that the captive breeding program should mix animals from multiple sites from the Guadarrama Mountain Range, but avoid the genetically most divergent populations.

INTRODUCTION

Maintenance of genetic diversity in populations is a major focus in conservation biology (Frankham *et al.* 2002). Risk of extinction due to genetic factors can be pronounced in small and isolated populations (Hartl & Clark 1997). As such, continual gene flow through population connectivity is crucial, especially in species with small population sizes or limited dispersal abilities (Frankham 2005). For these reasons, one of the global priorities of International Union of Conservation of Nature (IUCN) is the conservation of genetic diversity in fragmented and small populations (McNeely *et al.* 1990).

Amphibians are good biological models for investigating the genetic effects of fragmentation events because they typically show high levels of genetic differentiation at fine scales (Andersen *et al.* 2004, Richardson 2012, Trumbo *et al.* 2013). This may be due to their high level of philopatry (Semlitsch 2008), their limited dispersal abilities (Allentoft & O'Brien 2010), or to frequent local extinction-recolonization dynamics (metapopulation system; Wade & Mc Cauley 1988).

It is widely recognized that the rate of loss of amphibians around the globe is increasing (Allentoft & O'Brien 2010, Collins 2010). Of the 6.600 described species, 43% are currently threatened with extinction (Stuart *et al.* 2004). Pollution, climate change and emerging diseases are considered the major drivers behind the massive decline of amphibians (Collins 2010). The global emergence of diseases can lead to dramatic reductions in population size, especially when pathogen-induced mortality is additive (i.e., when pathogen-induced mortality is added to the normal mortality; Fisher *et al.* 2009, Tobler *et al.* 2012).

The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has affected amphibian populations on most continents, from tropical to temperate habitats with catastrophic consequences (Lips *et al.* 2006, Skerratt *et al.* 2007, Fisher *et al* 2009). Chytridiomycosis is a non-typical emerging disease with a broad host range and heterogeneous impacts on host populations (Kilpatrick *et al.* 2010). The reasons for its sudden emergence as an amphibian pathogen remain under intensive study (Collins & Storfer 2003, Collins 2010, 2013). Although there is still no clear general agreement about the relationship between the presence of *Bd* and environmental variables (Walker *et al.* 2010), studies have shown that

population level responses to infection can be explained by local environmental variables (Doddington *et al.* 2013).

The common midwife toad (*Alytes obstetricans*) is a widespread amphibian in central and western Europe with its southern range including the Iberian Peninsula. It is threatened over a great part of its distribution by the fragmentation and isolation of its populations (Bosch 2002) and more recently by the emergence of *Bd* (Bosch *et al.* 2001). Although recent studies have shown that populations can persist despite the enzootic presence of *Bd* under current environmental conditions (Tobler *et al.* 2012), *Alytes obstetricans* is one of the European amphibian species known to be highly susceptible to *Bd* (Bosch *et al.* 2001, Tobler & Schmidt 2010, Balaz *et al.* 2014). In Spain, the fungus has caused episodes of mass mortality in multiple species (*A. obstetricans, B. spinosus, S. salamandra*), mainly in montane areas (Bosch *et al.* 2001, Walker *et al.* 2010).

Our study was conducted at the index site for *Bd* in Europe: the Peñalara Massif and its surroundings (Bosch *et al.* 2001). The Peñalara Massif (formerly the Peñalara Natural Park and currently the heart of the Guadarrama National Park) is an alpine area at about 2.000 m of elevation in central Spain. The area has been protected for the past 70 years and, in spite of the high number of visitors (>100.000 per year), conservation and restoration practices maintain its ecological health in good condition. More than half of all ponds in this area are permanent, whereas the rest are temporary ponds. The Peñalara Massif has 10 amphibian species, of which *A. obstetricans* used to be one of the most abundant. In spring, *A. obstetricans* males formed large choruses in several locations, and reproduction was known to occur throughout the massif in the past (J. Bosch, personal observations). Midwife toads have a small clutch size and a remarkable reproductive behavior, as males carry the eggs twined around their hind legs on land for about a month, from fertilization to hatching. After hatching, larval development takes place in water bodies until metamorphosis is completed and juveniles leave the ponds. In the summers of 1997 and 1998, thousands of dead post-metamorphic *A. obstetricans* were found around the ponds of the Peñalara Massif. This situation prompted an intense survey in 1999, which led to the discovery of *Bd* as the responsible agent of this decline (Bosch *et al.* 2001). Within 5 years, the fungus caused the extirpation of around 90% of all populations, leaving only 8 breeding wild sites in the whole area. Although the species remains abundant in northern Spain, in Central Spain the situation is dramatic, especially in the Guadarrama Mountains Range. The neighboring

populations are located more than 40–50 km away, are quite small and not genetically related (Bosch 2002).

As a consequence of this outbreak of chytridiomycosis, and to avoid the complete extinction of the *A. obstetricans* in the Peñalara Massif, in 2008, the local government (Madrid, Consejería de Medio Ambiente, Vivienda y Ordenación del Territorio), the Spanish Museum of Natural History (CSIC) and the Durrell Wildlife Conservation Trust established a captive-breeding facility. The purpose of the captive breeding program is to maintain captive populations representative of the remaining genetic diversity, and to be a source for reintroductions back into the park. Twenty-one adults in total and some tadpoles were collected in 2008 from every remnant breeding site inside the Peñalara Massif and maintained in the facility in sterile conditions to avoid new *Bd* infection. At one site, Valdesquí (VQ), located outside the Peñalara Massif, individuals were collected in 1996 prior to the crash (60 tadpoles from a single temporary pond), and have been maintained in captivity since that time in a different location. Until the present study was concluded, all populations were kept separate to minimize possible outbreeding depression.

Here we evaluate the potential for captive stocks of *A. obstetricans* from the studied area to serve as a source of uninfected individuals for the reintroduction of this species into the Peñalara Massif. We used ten microsatellite markers to address three major questions: (1) Are the populations from the Peñalara Massif and surrounding areas genetically differentiated or do they form a single panmictic unit? (2) Can we detect signs of a genetic bottleneck in the extant populations of the *A. obstetricans* in the study area? (3) For future reintroductions, should we mix the present captive population before releasing them to the wild or should they be maintained separately?

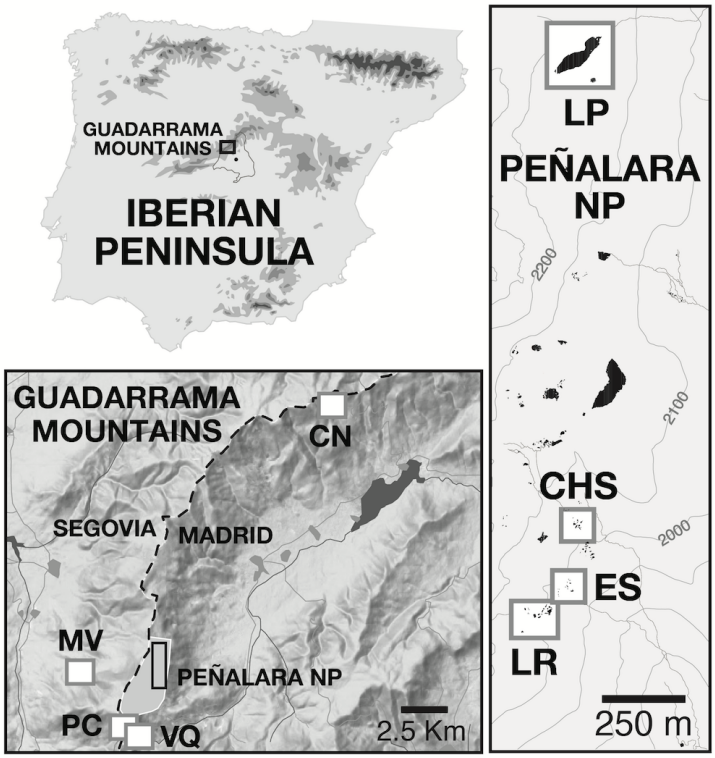

Figure 1. Location of the Peñalara Massif (formerly Peñalara Natural Park) in the center of the Guadarrama Mountain Range (Central Spain) in dark shading. A) Sampling sites outside the Peñalara Massifon the border between Segovia and Madrid: Circo del Nevero (CN), Montes de Valsaín (MV), Puerto de Cotos (PC) and Valdesqui (VQ). B) Sampling sites inside the Peñalara Massif: Laguna de Pájaros (LP), Charcas del Salto (ES), Charcas Secas (CHS) and Charcas de la Rubia (LR).

METHODS AND MATERIALS

Sample collection

We analysed a total of 106 individuals from the eight extant demes in the area of the Peñalara Massif. Here, a deme includes all individuals that use the same breeding site for reproduction. We included four demes inside the Peñalara Massif: Laguna de Pájaros (LP), Charcas del Salto (ES), Charcas Secas (CHS) and Charcas de la Rubia (LR), and four populations outside the massif: Puerto de Cotos (PC), Montes de Valsaín (MV) on the north side of the Sierra de Guadarrama, Valdesquí (VQ) and Circo del Nevero (CN), the most remote population, located about 20 km from the Peñalara Massif (Fig. 1, Table 1). For all sites except VQ, samples were

collected in 2009 in the field from larvae, or from individuals born in the wild but kept in the captive-breeding facility center. Samples from VQ were from individuals maintained in captivity since 1996. We collected tissue samples via tail clipping in the case of larvae and toe clips in the case of adults. All samples were stored in 70 % ethanol and maintained at -20 ºC.

Table 1. Parameters of genetic structure in eight populations of *Alytes obstetricans* for ten microsatellite loci.

Population	N	n_A	H_e	H_o	H	a_R	FIS	P_A
LP	20	3.9	0.571	0.653	0.588	3.125	-0.114	3(2)
VQ	13	2.7	0.484	0.517	0.504	2.498	-0.024	0
PC	6	3.5	0.582	0.617	0.637	3.500	0.031*	1
ES	11	2.7	0.404	0.340	0.436	2.474	0.210**	0
CHS	8	2.7	0.485	0.529	0.517	2.607	-0.013	1
LR	17	4	0.485	0.430	0.581	3.303	0.165**	1
CN	20	3	0.415	0.395	0.426	2.610	0.073	1
MV	11	4.6	0.645	0.609	0.679	3.982	0.102**	6 (6)
Average						0.60	3.08	0.020
SE						0.08	0.4	0.056

Sample size (N), average number of alleles per locus (n_A), expected heterozygosity (H_e), observed heterozygosity (H_o), gene diversity (H), allelic richness (a_R), inbreeding coefficient (FIS) and number of private alleles per locality (P_A, number of loci). Asterisks denote significant values: * p\0.05, ** p\0.01

DNA EXTRACTION AND PCR AMPLIFICATION

DNA was extracted using the QIAGEN DNeasy Tissue Extraction kit. We used 10 polymorphic microsatellites developed for *A. obstetricans* (Tobler et *al.* 2013) to characterize the genetic structure of the populations of Peñalara Park. Individual loci were PCR amplified in a final volume of 20 µl containing 1X PCR buffer [67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20], 2.5 mM $MgCl_2$, 0.01 % BSA (Roche Diagnostics), 0.25 µM dNTPs, 0.40 µM dye-labelled M13 primer, 0.25 µM reverse primer, 0.034 µM M13 tailed-forward primer, 0.5 U *Taq* DNA polymerase (Bioline) and 5 µl of genomic DNA. A sequence tail 5´-GTTTCT-3´ was added to the 5´ end of the reverse primer to improve adenylation and facilitate genotyping (Brownstein *et al.* 1996).

Samples were amplified in a 'touchdown' PCR in a BIO-RAD DNA Engine Peltier Thermal Cycler, with an initial 2 min of denaturation at 94 ºC; 17 cycles at 92 ºC for 30 s, annealing at 60–44 ºC for 30 s (1 ºC

decrease in each cycle) and extension at 72 °C for 30 s; 25 cycles of 92 °C for 30 s, 44 °C for 30 s and 72 °C for 30 s with a final extension for 5 min at 72 °C. Amplified fragments were analyzed on an ABI 3130xl Genetic Analyser and scored using GENE MAPPER 4.0 (Applied Biosystems) and LIZ 500 size standard.

Population genetic differentiation and genetic diversity

Departure from Hardy–Weinberg equilibrium for each locus in each of the eight studied breeding sites was calculated using a test analogous to Fisher's exact test in GENEPOP 4.0 (Rousset 1997). Microsatellite diversity indices, average number of alleles per locus, observed heterozygosity (H_o) and expected heterozygosity (H_e) were computed from allele frequencies under the assumption of random mating using GENETIX 4.03 (Belkhir *et al.* 2001). Allelic richness and the population-inbreeding coefficient (F_{IS}) were calculated with FSTAT 2.9.3 (Goudet 2001).

Population structure was evaluated with the unbiased estimator of Wright's (1951) F_{ST} using GEN ALEX 6.2 (Peakall & Smouse 2006). Partitioning of genetic variance (AMOVA) among individuals and populations was calculated with the same software. Isolation by distance was assessed in IBDWS 3.16 (http://ibdws.sdsu.edu/~ibdws/) by testing the correlation between the logarith of pairwise geographical distances (calculated from longitude and latitude data (http://www.chemical-ecology.net/java/lat-long.htm)) and F_{ST} /(1–F_{ST}) values.

We also examined population genetic structure using the model-based approach implemented in STRUCTURE 2.3.2 (Pritchard *et al.* 2000). Using a Bayesian approach, STRUCTURE estimates the number of genetic clusters without prior information on geographic clusters and assigns a posterior probability to each individual of belonging to each of K inferred clusters. We used the admixture model with correlated allele frequencies and the maximum number of the clusters was 11 (K = 1–11). For each K we ran 20 replicates of 1.000.000 iterations of Markov chain Monte Carlo with burn-in periods of 200.000 iterations. We applied the Evanno *et al.* (2005) method to estimate the most likely number of genetic clusters (ΔK).

BOTTLENECK TEST

To evaluate the evidence for recent bottleneck events, we used three different approaches. First, we assessed the effect of recent and severe population reduction using a one-tailed Wilcoxon signed rank test (10.000 iterations) to determine if observed heterozygosity was higher than expected under mutation-drift equilibrium for the observed number of alleles, as implemented in the software BOTTLENECK1.2.02 (Piry *et al.* 1999). The Wilcoxon test is the most appropriate and powerful test when less than 20 loci are used (Piry *et al.* 1999). The distributions of expected heterozygosities were estimated under three models of microsatellite mutations: the two extreme models of the stepwise mutation model (SMM) and the infinite allele model (IAM), and the intermediate two-phase model (TPM) with the default settings of 30% mutations under IAM and 70% under SMM. Second, we used a mode shift test to detect distortion of the L-shape expected under equilibrium for the frequency distribution of allele classes for each of the eight populations (Luikart *et al.* 1998). In the third method, we calculated the statistical significance of the M statistic in each population using the *M-ratio* (M), as implemented in the software M_P_Val (Garza & Williamson 2001). In this test, M is the ratio between the number of alleles at a locus and the total range of allele sizes. M tends to be smaller in recently reduced populations than in equilibrium populations (Mc) because the range in allele size is expected to decrease less than the number of alleles after a reduction in population size (Garza & Williamson 2001). The critical values of M for different pre-bottleneck scenarios (Mc) were calculated using the software CRITICAL_M (Garza and Williamson 2001). We used the default parameters suggested by Garza and Williamson (2001); μ (microsatellite mutation rate) = 5.0×10^{-4} /locus/generation, Δ_g (average repetition frequency of multi-step mutation) = 3.5, and P_g (proportions of multi-step mutations) = 0.22 according to the suggestions of Peery *et al.* (2012) in order to avoid type I error. For the calculation of θ, which can vary depending on Ne ($\theta = 4Ne\mu$), we used Ne values of 50, 100, 500, and to 1.000 individuals per population, based on direct estimates of the individuals in the pond previous to the *Bd* mortality episode, using the maximal and the minimal number of counted individuals (J. Bosch, personal observations).

RESULTS

Genetic diversity and population structure

We detected a total of 77 alleles in the 10 loci across all sampled demes of *A. obstetricans*, with individual loci ranging from 4 to 19 alleles. A few loci showed significant deviations from Hardy–Weinberg equilibrium after Bonferroni correction in two breeding sites: four loci in population LR (Aobs8, Aobs28, Aobs25 and Aobs17) and one locus in population MV (Aobs 8). Genetic diversity parameters appear in Table 1. Overall Fst value was 0.252 (SE = 0.023). Estimates of pairwise Fst ranged from 0.093 to 0.447, and all pairs of breeding sites were significantly differentiated ($p < 0.05$) (Table 2). Four of the eight breeding sites showed significant positive F_{IS} values (PC, ES, LR, MV) indicating heterozygote deficits (Table 1).

Table 1. Population differentiation among the midwife toad localities

	LP	VQ	PC	ES	CHS	LR	CN
LP	–	**0.204**	**0.149**	**0.276**	**0.177**	**0.165**	**0.328**
VQ	4.88	–	**0.178**	**0.292**	**0.282**	**0.212**	**0.435**
PC	4.26	0.64	–	**0.197**	**0.180**	**0.187**	**0.282**
ES	1.60	3.60	2.75	–	**0.261**	**0.093**	**0.473**
CHS	1.38	3.54	2.95	0.21	–	**0.208**	**0.410**
LR	1.66	3.26	2.67	0.06	0.22	–	**0.447**
CN	16.86	21.43	20.90	18.17	17.99	18.26	–
MV	6.14	7.55	6.56	6.39	6.33	6.34	20.98

Mesaured by pairwise F_{ST} (above diagonal), and geographic distances in Km between pairs of populations (below diagonal). Bold numbers indicate values significantly different from zero ($\alpha<0.05$)

The AMOVA analysis indicated that 61% of the molecular variation occurred within breeding sites and 39 % between breeding sites. We found evidence for isolation by distance based on a significant Mantel test between genetic and geographic distances when all populations were considered ($R^2 = 0.3618$, $F_{1, 27} = 14.739$, $p = 0.0007$), however significance was lost when we eliminated comparisons involving the most isolated and differentiated site CN ($R^2 = 0.0494$, $F_{1, 20} = 0.9879$, $p = 0.3246$). The factorial correspondence analysis corroborated that the breeding site CN was the most distinct from all other breeding sites. This analysis also revealed high genetic differentiation of the individuals in the breeding site MV located on the opposite side of the mountain range (Fig. 2). When K was set to 2 during the clustering analysis performed with STRUCTURE,

the CN deme was differentiated from the rest of the demes (data not shown). Applying the ΔK method of Evanno *et al.* (2005) we consistently obtained estimates of the highest likelihoods for the models with K = 6 across independent runs. Under K = 6, VQ, ES, CHS, and CN were all distinct clusters, while MV and PC were pooled in a single cluster (Fig. 4). LP was distinct from the remaining sites, but had evidence of some recent gene flow from VQ. LR was the most admixed site, being largely similar to ES but with some gene flow from CHS and PC/MV. The remaining three clusters identified by STRUCTURE corresponded to the other three breeding sites included in the analysis (LP, VQ and CHS), with some evidence for recent gene flow from VQ to LP (Fig. 4).

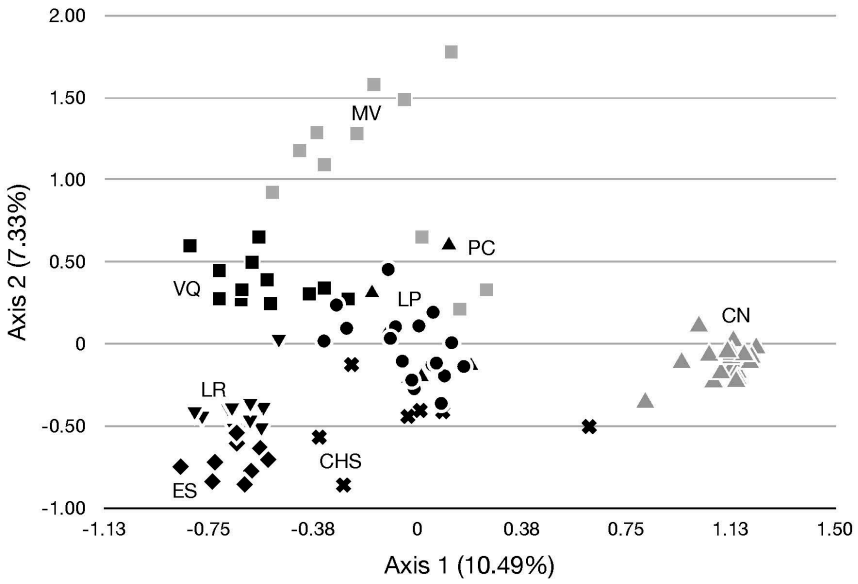

Figure 2. Two-dimensional graph based on Factorial Component Analysis, illustrating the relationship between *A. obstetricans* individuals in the Guadarrama Mountains. The first two dimensions are shown with the percentage of variation described noted in parentheses. The different symbols correspond to the different study populations.

BOTTLENECK TESTS

Most breeding sites showed evidence of past population contraction, although no consistent evidence of population bottleneck was found across the three methods used. Results of the analysis with BOTTLENECK depended on the mutation model assumed: the infinite allele model (IAM) was significant for historical reduction in six of the eight populations, the

two-phase mutational model (TPM) was only significant for LP and VQ, whereas the stepwise mutational model (SMM) did not show any significant results (Table 3). In addition, the mode shift test supported a population bottleneck in half of the analyzed populations (VQ, PC, CHS and CN). On the other hand, the third method, *M-ratio* was mostly consistent across populations, and indicated population contractions with values of M < Mc for the assumed prebottleneck Ne values tested of 50 and 100. When these Ne values were larger (500 and 1.000) the results did not support a recent population contraction in ES, LR and CN (Table 2).

DISCUSSION

Genetic structure and population contraction

Despite the short geographical distances between most of the ponds (a few kilometers) and the absence of apparent geographic barriers, dispersal between some of the studied breeding sites appeared to be limited. We found strong indication of isolation by distance when the farthest population CN was included in the analysis. Interestingly, that signature disappeared when this population was eliminated from the analysis (Fig. 3). Isolation by distance is an expected equilibrium pattern under limited dispersal and is commonly observed in amphibian metapopulations due to their poor dispersal abilities (Funk *et al.* 2005, Spear *et al.* 2005, Wang & Summers 2010). The absence of such a pattern within the focal area might be interpreted as an additional signal of recent local drift eroding the expected equilibrium pattern. Additionally, the Bayesian clustering analysis implemented in STRUCTURE supports the result of isolation by distance.

On the other hand, LR and LP showed some degree of admixture with other populations. Probabilities of population membership indicate the presence of some first generation immigrants and crosses between residents and immigrants, which may indicate sporadic dispersal events. A more striking case of genetic exchange is provided by the single cluster formed by the populations MV and PC, which are separated by 6.56 km (Fig. 4). These results are also supported by low F-statistic differentiation (see Table 2). One possible explanation could be the type of water bodies in this area. The small size, short hydroperiod and shortage of ponds in the basal areas of the mountains where these populations are located, might be

inducing the dispersal of individuals in search of better breeding sites (Fig. 1).

Table 2. Results (P-values) of the three bottleneck tests

BOTTLENECK				Mode-shift	*M-ratio* (Mc)				
Pop	TPM	IAM	SMM		M	θ = 0.1	θ = 0.2	θ = 1	θ = 2
LP	**0.042**	**0.001**	0.347	–	0.644	**0.754**	**0.740**	**0.672**	**0.751**
VQ	**0.042**	**0.003**	0.080	+	0.508	**0.756**	**0.736**	**0.669**	**0.626**
PC	0.246	0.080	0.615	+	0.559	**0.753**	**0.737**	**0.661**	**0.599**
ES	0.179	0.064	0.673	–	0.642	**0.750**	**0.738**	**0.663**	0.618
CHS	0.138	**0.012**	0.278	+	0.461	**0.751**	**0.740**	**0.664**	**0.611**
LR	0.384	**0.012**	0.883	–	0.685	**0.752**	**0.742**	0.671	0.629
CN	0.191	**0.013**	0.472	+	0.688	**0.754**	**0.740**	0.671	**0.751**
MV	0.187	**0.012**	0.577	–	0.574	**0.750**	**0.738**	**0.663**	**0.618**

Results are based on ten microsatellite loci for eight populations of *Alytes obstetricans*. Analyses with BOTTLENECK used three models microsatellite mutation: two-phase mutation (TPM), infinite alleles (IAM) and stepwise mutation (SMM). For the Mode-shift test, modes are indicated by - for normal L-shaped and + for shifted mode. Observed *M-ratio* (M) and critical ratio (Mc) estimated for each of four values of pre-botleneck θ that correspond to a four values of Ne respectively (50, 100, 500, 1.000). Bold numbers indicate values significantly different from zero (α < 0.05) in the Bottleneck analysis and a population reduction in size when M < Mc in *M-ratio* analysis

In contrast, the general pattern of high genetic differentiation between nearby permanent ponds (as the case of PC and VQ) may indicate that these ponds are high quality habitat and thus individuals remain associated with the pond, that the nature of the surrounding terrestrial habitat limits migration, or, alternatively, that immigrants are less successful breeders than resident toads. Furthermore, small sample sizes could have biased our results and lead to the extreme genetic differentiation of ES and CHS despite their geographic proximity.

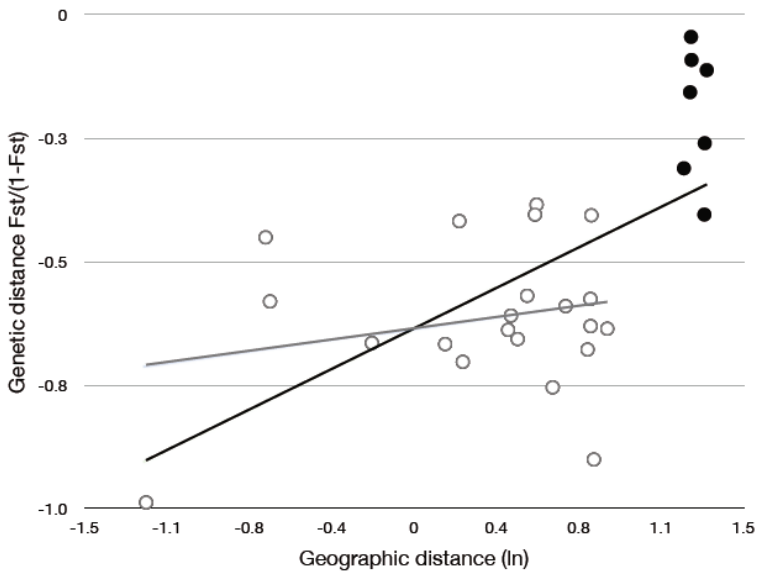

Figure 3. Isolation by distance analyses representing the relationships between pairwise values of Fst/(1-Fst) and logarithm of geographical distance between sites. The linear curve represents the isolation by distance pattern observed ($p < 0.05$) including the population CN (*black dots, black line*) and without CN (*white dots, grey line*)

Population bottleneck analyses indicated reductions in genetic diversity in some populations under some mutation models and under the range of population sizes tested. In some populations, a bottleneck was inferred under IAM but not SMM, despite SMM being the more realistic mutation model when microsatellites are being used (Di Rienzo *et al.* 1994). The significance of the heterozygosity excess test is highly dependent on the assumed mutational model, so the results should be interpreted with caution. A small number of markers might not provide adequate resolution under the SMM model; however we used 10 microsatellite loci, which following Luikart & Cornuet (1998) is enough to achieve sufficient statistical power. In contrast, the *M-ratio* approach detected a persistent bottleneck signature in all populations analyzed under all values of θ. Despite this finding, the interpretation of *M-ratio* results must be done with caution because we do not have specific information on either the mutation rate (μ) of our microsatellite set or on the average size of non-single step mutations (Δ_g), both of which can affect the *M-ratio* (Garza and Williamson 2001). Furthermore, when we varied the mutation rate (changing θ), the results were largely congruent with the heterozygosity excess test.

Although, the *M-ratio* and BOTTLENECK test measures different properties (in terms of duration and severity) of the bottleneck, the results may not be contradictory. Studies have shown that while *M-ratio* is more likely to identify a bottleneck that occurred in the distant past with severely reduced population size, BOTTLENECK will more frequently detect a weak or moderate bottleneck that occurred recently (Williamson-Natesan 2005). Thus, our results suggest that bottlenecks in the *A. obstetricans* populations occurred in the recent past and were fairly severe, which is consistent with the observed massive decline of the populations due to chytridiomycosis, but does not imply direct causation.

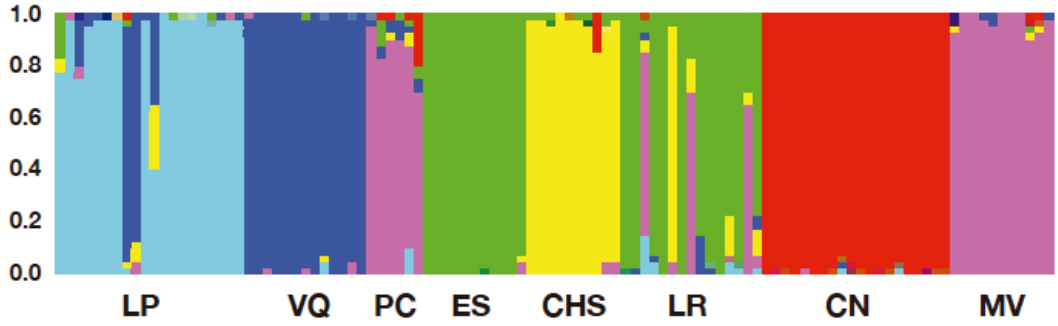

Figure 4. Bayesian clustering analysis of *A. obstetricans* breeding sites in the Guadarrama Mountains using STRUCTURE. Each vertical line represents one individual, and color proportion shows individual membership coefficients. Colors represent different genetic clusters and letters show the breeding sites (see Fig. 1 for full names). The y-axis represents individual assignment probabilities

Conclusion and conservation implications

In the case of *A. obstetricans*, 90% of the populations in the Peñalara Massif disappeared after the emergence of the disease chytridiomycosis (Bosch *et al.* 2001) and the entire population has remained below 100 individuals ever since. It has been argued that effective population sizes of <100 individuals can negatively affect the fitness and viability of populations (Lande 1998). Recently, congenital defects in the forelimbs and bones of the spine have been observed in captive individuals of midwife toads from Valdesquí (J. Bosch personal observation). These deformities may be early signs of inbreeding depression, a problem, which has been identified as one of the most frequent causes of the failure of reintroduction and recovery programs (Spielman *et al.* 2004a, Frankham 2005). Especially when captive breeding is necessary, the appropriate selection of the source of founder individuals for future reintroductions

may determine the success of the program. For these reasons, genetic studies prior to the management of threatened vertebrate populations have increased in number (Seddon *et al.* 2007), including for the conservation of endangered amphibians (Kraaijeveld-Smit *et al.* 2005, Vredenburg *et al.* 2007, Beauclerc *et al.* 2010). When populations are isolated and there are signs of inbreeding depression, genetic rescue can be achieved through induced migration (Moritz 1999). While this should be preferentially done with populations of the same genetic lineages in order to prevent inbreeding depression, in some cases the mixture of genetic lineages has been used when this first option is not feasible (Godoy *et al.* 2004, Beauclerc *et al.* 2010). Many authors have shown that by mixing lineages it is possible to obtain rapid population growth, an increase in heterozygosity, and a quick spread of new alleles (Madsen *et al.* 2004, Vilà *et al.* 2003, Johnson *et al.* 2010). This is especially important when populations are found in stressful environments, for example, when they must deal with a novel pathogen (Spielman *et al.* 2004b). On these grounds and for future reintroductions we recommend mixing populations using individuals of all remnant populations in the area as founders of the captive breeding program, with the possible exception of CN, a population that is most geographically separated and that showed evidence of lack of recent gene flow with Peñalara Massif. In contrast, as individuals from the higher elevations of the Peñalara Massif (LP, CHS, ES, LR) do share alleles with animals at lower elevations (PC, VQ, MV), we recommend treating all these populations as a single conservation unit. That strategy will help to recover the lost genetic diversity in the wake of the fungus disease and to maximize the short- and long-term viability of midwife toads in this area.

In summary, *A. obstetricans* in Guadarrama Mountains showed evidence of a population bottleneck, limited genetic diversity and strong genetic population structure among breeding sites. This translates into multiple genetic units with non-random mating between sites. Although low dispersal and geographical barriers can produce this pattern we suggest disease-induced population decline may also be a contributing factor. The genetic bottleneck observed and low genetic variability in these small and isolated populations indicates that they are at high probability of local extinction.

In addition, the high genetic subdivision among populations across very short geographic distance suggests low capacity for re-colonization of nearby ponds following local extinction events, and therefore, continued management should include artificial reintroductions.

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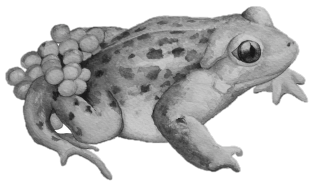

CAPÍTULO 7

HETEROGENEIDAD EN LA INFECCIÓN Y TRANSMISIÓN DE UN PATÓGENO MULTI-HOSPEDADOR (*BATRACHOCHYTRIUM DENDROBATIDIS*) EN UNA COMUNIDAD DE ANFIBIOS

INFECTION AND TRANSMISSION HETEROGENEITY OF A MULTI-HOST PATHOGEN (BATRACHOCHYTRIUM DENDROBATIDIS) WITHIN AN AMPHIBIAN COMMUNITY

HETEROGENEIDAD EN LA INFECCIÓN Y TRANSMISIÓN DE UN PATÓGENO MULTI-HOSPEDADOR (*BATRACHOCHYTRIUM DENDROBATIDIS*) EN UNA COMUNIDAD DE ANFIBIOS

RESUMEN

La mayoría de los parásitos infectan mútiples hospedadores. Como el resultado de la infección es diferente en cada uno de ellos, la mayoría de los estudios de enfermedades de fauna silvestre se centran en las pocas especies que sufren las consecuencias más severas. No obstante, el rol que cada hospedador puede desempeñar en la persistencia y transmisión de la infección puede ser crucial para entender la expansión del parásito y el riesgo que supone para la comunidad. Las teorías actuales predicen que ciertos hospedadores pueden modular la infección de otras especies amplificando o diluyendo tanto la intensidad como la prevalencia de la infección, lo cual tiene implicaciones en el riesgo de contraer la enfermedad dentro de las comunidades. El hongo *Batrachochytrium dendrobatidis* (*Bd*), el agente causal de la quiridiomicosis, ha provocado declives globales de poblaciones de anfibios y extinciones. Sin embargo, no afecta a todas las especies por igual y por ello, *Bd* es un buen ejemplo de un patógeno multi-hospedador que debe ser estudiado bajo la perspectiva de toda la comunidad. Para analizar como el sapo partero común (*Alytes obstetricans*) es un reservorio y un posible amplificador de la infección en otras especies utilizamos una aproximación experimental en poblaciones cautivas y silvestres para determinar el efecto de la presencia de larvas de sapo partero común sobre la infección de otras especies que conviven con el en el Macizo de Peñalara, España. Observamos que la especie más severamente afectada, el sapo partero común, puede estar amplificando la carga de infección de las otras especies, todas ellas con diferente susceptibilidad a la infección por *Bd*. Nuestros resultados tienen importantes implicaciones para llevar a cabo acciones de mitigación centradas en el hospedador potencialemente "amplificador" y para entender mejor los mecanismos de transmisión de *Bd.*

INFECTION AND TRANSMISSION HETEROGENEITY OF A MULTI-HOST PATHOGEN (*BATRACHOCHYTRIUM DENDROBATIDIS*) WITHIN AN AMPHIBIAN COMMUNITY

ABSTRACT

The majority of parasites infect multiple hosts. As the outcome of the infection is different in each of them, most studies of wildlife disease focus on the few species that suffer the most severe consequences. However, the role that each host plays in the persistence and transmission of infection can be crucial to understanding the spread of a parasite and the risk it poses to the community. Current theory predicts that certain host species can modulate the infection in other species by amplifying or diluting both infection prevalence and infection intensity, both of which have implications for disease risk within those communities. The fungus *Batrachochytrium dendrobatidis* (*Bd*), the causal agent of the disease chytridiomycosis, has caused global amphibian population declines and extinctions. However, not all infected species are affected equally, and thus *Bd* is a good example of a multi-host pathogen that must ultimately be studied with a community approach. To test whether the common midwife toad *Alytes obstetricans* is a reservoir and possible amplifier of infection of other species, we used experimental approaches in captive and wild populations to determine the effect of common midwife toad larvae on infection of other amphibian species found in the Peñalara Massif, Spain. We observed that the most widely and heavily infected species, the common midwife toad, may be amplifying the infection loads in other species, all of which have different degrees of susceptibility to *Bd* infection. Our results have important implications for performing mitigation actions focused on potential 'amplifier' hosts and for better understanding the mechanisms of *Bd* transmission.

INTRODUCTION

The majority of parasites are able to infect multiple hosts (Fenton & Pedersen 2005). For example, most human pathogens are zoonotic in origin, and the majority of pathogens of livestock and domesticated species originated in wildlife species (Daszak *et al.* 2000). However, even within the widest host base, there exists a great deal of variation in how frequently and heavily different species become infected (Fenton & Pedersen 2005). As a result, host species play different roles in the persistence and transmission of infection within a community. Just as individual-level transmission is highly skewed towards certain key individuals (Lloyd-Smith *et al.* 2005), the presence of certain species within a host community can be disproportionately important in the success of parasite invasion and persistence (Rudge *et al.* 2013). There are a number of ways in which a species may be of particular importance.

'Vectors', 'reservoirs', 'amplifiers' and 'diluters' of infection are all terms used to describe species that, in different ways, help maintain, spread or reduce infection within a community. While vectors and reservoirs are widely accepted concepts, empirical evidence for the existence of pathogen amplification or dilution by hosts in natural systems is comparatively more limited (but see Searle *et al.* 2011). By definition, amplification hosts are species that make a pathogen more likely to persist and more abundant than it would be in the absence of that species (Begon 2008). By increasing the overall prevalence and infection intensity within sympatric species, amplification hosts may increase the risk of disease emergence within a host assemblage. Quantifying species' differences in host competence and their roles in parasite transmission is therefore essential if we are to understand the dynamics of infection and the likelihood of disease emergence within a community.

The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is a host-generalist pathogen that infects mainly the keratinized skin of developed amphibians and the mouthparts of amphibian larvae (Berger *et al.* 1998). Chytridiomycosis is an amphibian-specific emerging infectious disease caused by the fungus, and it has caused severe population declines and species extirpations and extinctions worldwide (Stuart *et al.* 2004). It is known to have infected over 400 species, including species in all 3 amphibian Orders (Gower *et al.* 2013; see also www.bd-maps.net), and probably many more species are susceptible to *Bd* infection. However, there is a great deal of variation in the pathogenic effects of *Bd* both among (Lips

et al. 2006, Bielby *et al.* 2008) and within species (Walker *et al.* 2010). A range of intrinsic (Woodhams *et al.* 2007, Farrer *et al.* 2011, Jani & Briggs 2014) and extrinsic factors (Vredenburg *et al.* 2010, Raffel *et al.* 2015) have been linked to the variation in the effect of *Bd* on species and communities, but, as yet, relatively little is known about the role of community composition in this context.

The first known chytridiomycosis-related mortalities in Europe occurred in the late 1990s and led the common midwife toad *Alytes obstetricans* (*Ao*) to the brink of local extinction in the Peñalara Massif at the Sierra de Guadarrama National Park (Bosch *et al.* 2001). The species seems to be the most severely impacted species in Europe (Tobler & Schmidt 2010, Walker *et al.* 2010), and as a result of the ease with which it becomes infected, the clade Alytidae acts as a reliable sentinel when screening for infection in new regions or populations (Baláž *et al.* 2014). However, other species in heavily affected assemblages, such as those in Guadarrama, exhibit a variety of responses to *Bd* exposure. Following initial *Ao* mass mortalities, common toads *Bufo spinosus* and fire salamanders *Salamandra salamandra* also suffered mortality and declines as a result of chytridiomycosis (Martínez-Solano *et al.* 2003, Bosch & Martínez-Solano 2006, Bosch *et al.* 2014). In contrast, the rest of the amphibian species within the community at Guadarrama seem not to have been seriously affected by the disease, although all of them can be infected by the pathogen (www.bd-maps.net). The population-level effects of chytridiomycosis on *B. spinosus* and *S. salamandra* diminished following the near extinction of *Ao* (J. Bosch unpubl. data) and there fore we hypothesize that *Ao* was driving much of the infection transmission.

To better understand the risk of disease emergence within a host community, it is important to understand how different species within that community differ in their susceptibility to *Bd* infection, and their role in infection transmission. In this study, we chose to look at the range of tolerance to *Bd* across the amphibian species of the Peñalara Massif and investigate aspects of the transmission of the pathogen within this assemblage. Doing so is important for designing better management strategies, preventing future declines and improving reintroduction programme success. Specifically, we tested the hypotheses that *Ao* acts as an amplification host and that individuals of sympatric species will experience a higher probability and greater intensity of infection than individuals housed only with their own species. Further, we tested the hypothesis that *Bd* can transmit both directly, from *Ao* larvae to sympatric species, and also

indirectly through swimming zoospore transmission, rather than relying on direct contact with an infected host. Finally, we investigated whether community members (i.e. individual species) exhibit different levels of infection from one another when housed with *Ao* larvae. Combined, these experiments may help to explain some of the observed community-level impacts of chytridiomycosis in the presence and absence of *Ao*.

METHODS AND MATERIALS

The Peñalara Massif is home to 8 endemic amphibian species: *A. obstetricans*, *Bufo calamita*, *B. spinosus*, *Hyla molleri*, *Pelophylax perezi*, *Rana iberica*, *Triturus marmoratus* and *Salamandra salamandra*. Additionally, *Ichthyosaura alpestris* was recently introduced in the area (Martínez-Solano *et al.* 2003).

Bd sampling

To analyse *Bd* infection loads, we took samples when the larvae were judged to be close to metamorphosis from the parts of the body where the concentration of zoospores is generally highest (Garner *et al.* 2009): the hind limbs of anurans or the complete body of urodeles. As *Ao* tadpoles have a longer developmental time (up to 5 yr), samples were taken from the keratinized mouthparts when companion species were sampled, or at the end of the experiment. Samples were taken from live animals with a fine-tipped sterile swab (Medical Wire and Equipment 113) or directly from the corresponding tissue of newly dead individuals or after euthanasia.

Laboratory methods

To quantify infection load in amphibians, we used a quantitative real-time polymerase chain reaction (qPCR) protocol (Boyle *et al.* 2004). Extractions were diluted 1:10 before real-time PCR amplification, performed in duplicate, with Bd genomic equivalent (GE) standards of 100, 10, 1 and 0.1 GE (isolate IA042, Ibón Acherito, Spanish Pyrenees) in a CFX96 machine (BioRad). When only 1 replicate from any sample amplified, we assayed this sample a third time. If the third amplification did not result in an amplification profile, we considered the sample negative for infection.

Experiment 1

This experiment was set up to test the hypothesis that *Ao* acts as an amplification host by increasing infection prevalence and intensity in sympatric species, and also that *Bd* can initially infect hosts indirectly through waterborne spores, rather than relying on direct contact with an infected host. We conducted a field experiment in the Laguna Grande de Peñalara glacial lake of the Peñalara Massif (2.018 m.a.s.l.), and *B. spinosus* was chosen as our focal susceptible species as it has been observed to suffer infection and mortality as a result of *Bd* infection in natural surroundings (Bosch & Martínez-Solano 2006) and in experimental settings (Garner *et al.* 2009). Several hundred *B. spinosus* free-swimming Gosner stage 25 tadpoles (Gosner 1960) were collected from different locations at Laguna Grande to average any possible genetic variation among offspring. Previous studies have shown that at this stage of development, *B. spinosus* tadpoles lack *Bd* infection (Ortiz-Santaliestra *et al.* 2011). Uninfected Ao larvae were obtained from a captive colony located in the studied area that is regularly tested for *Bd* infection by qPCR. Larvae from the stock of our focal species, *B. spinosus*, were assigned to 1 of 4 different treatments in a 2 x 2 experimental design. The 2 factors of interest were density and the presence of *Ao* larvae, and each of these 2 factors had 2 levels: high density (50 *B. spinosus* larvae) or low density (25 *B. spinosus* larvae), and presence (10 larvae) or absence (0 larvae) of *Ao* larvae. The selected densities are within the range typically observed naturally in this system (J. Bosch unpubl. data). Each treatment was replicated 3 times, each group being housed in a separate 4 l container. The containers had ventilated sides and were placed together floating in the lake. Water temperature inside each container was re corded with a thermocouple thermometer in a randomized order and found not to differ between containers. The experimental design removed the possibility that infection was introduced with any of the experimental animals, as they came from uninfected stock, or were placed in the experiment before keratinized mouthparts had developed and had the opportunity to become infected. Instead, experimental animals could only become infected when ex posed to zoospores in the lake water. Once the most advanced *B. spinosus* tadpoles were close to metamorphosis (31 d after the experiment began), the experiment was ended, and we euthanized 20 randomly selected *B. spinosus* tadpoles (Gosner stages 38–42) per container and stored them in 70% ethanol before processing for *Bd* infection. We ended the experiment at this point because we wanted to assess infection in larvae, before they undergo metamorphosis when some individuals lose infection, or infection becomes difficult to detect (Garner *et al.* 2009).

To see whether the 4 experimental levels resulted in a different probability of infection, we used a chi-squared test, and, in the presence of any significant variation, we used generalized linear models (GLMs) with binomial errors to determine which of the 2 factors best explained variation in infection probability of *B. spinosus*. For the latter analysis, backwards-stepwise regression of a full model including all terms was implemented, with changes in model fit being measured using analysis of deviance. Because our experimental design does not adequately account for the total density of larvae when considering the presence and absence of *Ao* as a factor (i.e. within each level of the density treatments, *B. spinosus* had different total tadpole densities depending on whether *Ao* was present), we used binomial tests to identify whether the proportion of individuals infected significantly varied in the high and low density treatments in the absence of *Ao*. Doing so allowed us to determine whether an increase in the density of the focal host was an important factor in infection levels in the absence of *Ao*.

To analyse whether infection intensity varied with density and presence/absence of *Ao* larvae, we used GLMs with negative binomial errors using the glm.nb function from the R package MASS. The function glht from the multcomp library was used to determine which levels of the 4 treatments varied from one another.

Experiment 2

This experiment tested whether species co-housed with *Ao* larvae differed from one another in probability and intensity of infection. Plastic containers (2 l capacity, n = 52) were floated together in the Laguna Grande de Peñalara. The containers had holes to allow water exchange with the surrounding lake. Water temperature inside the containers was measured with a thermocouple thermometer in random order without mixing the water before sampling began, and it did not differ significantly among containers. Each of 13 treatments was replicated 4 times. The 13 treatments were (1) 2 larvae of *Ao* alone, which acted as a control to see how heavy infection was in this species when housed alone; (2–7) 6 treatments consisting of 2 larvae of *Ao* co-housed with 2 larvae of each of *B. spinosus, B. calamita, H. molleri, P. perezi, R. iberica or S. salamandra*; and (8–13) 2 larvae of each of those 6 species alone (i.e. no *Ao* were added). Larvae of the studied species were collected in the field in several ponds of the Peñalara Massif, and *Ao* larvae were obtained from the captive colony. All larvae were placed in the experimental set-ups at an early stage of their

development before keratinized mouthparts had developed, and their uninfected status was confirmed by qPCR. One overwintered larva of *S. salamandra* from the same lake was introduced into each container for 1 wk. As overwintered larvae, *S. salamandra* have an infection prevalence of 100% in spring in this system (Medina *et al.* 2015). Therefore this was a guaranteed way to expose experimental animals to infection regardless of whether experimental animals were exposed to zoospores in the lake water. At the end of the experiment, we measured the infection intensity of all larvae in each of the 13 treatments.

We tested whether species differed from one another in their infection probability. Using a Fisher's exact test, we determined whether the proportion of infected individuals of the different species varied, and in the event of a significantly non-random distribution of infection, we used binomial tests to determine which species varied significantly from the background prevalence of infection in the experiment.

To determine whether infection intensity in cohoused species was higher in the presence of *Ao*, we conducted a Student's *t*-test to compare infection intensity between those individuals co-housed with Ao with those housed only with a conspecific for each of the 6 co-housed species.

To investigate whether infection intensity differed among each species when co-housed with *Ao*, we used a GLM with negative binomial errors and Tukey comparisons. The same statistical tests were used to determine whether *Ao* varied in infection intensity when co-housed with different species. GLMs with negative binomial errors were conducted using the glm.nb function from the MASS library, and the Tukey comparisons on the resulting glm.nb object were made using the glht function from the multcomp library.

Experiment 3

The following experimental set-up in the laboratory was used to test whether *Ao* can transmit *Bd* infections directly to other species, whether those species differ from one another in the resulting infection intensity, and whether *Ao* experiences different levels of infection when co-housed with other species. Newly hatched larvae of 5 species (*H. molleri, P. perezi, I. alpestris, T. marmoratus and S. salamandra*) were captured in the field in several ponds of the Peñalara Massif, and their uninfected status was confirmed by qPCR. Two larvae of each of these species were placed in the

presence of a single infected *Ao* larva, resulting in 5 experimental treatments. The sixth treatment was a single infected Ao larva, housed alone. Each of the 6 treatments was replicated 10 times in 2011 and 15 times in 2012. All experimental replicates were housed in 1.5 l containers maintained at a temperature of 18°C. All *Ao* larvae were collected from a well-studied population (Toro, Zamora, western-central Spain; Fernández-Beaskoetxea *et al.* 2015), and their infection status was checked by qPCR before the experiment started. To test whether species differed from one another in their probability of infection, we used a Fisher's exact test to compare proportions of infected and uninfected individuals for each species. To test whether species differed from one another in their infection intensity when co-housed with *Ao* larvae, we used a GLM with negative binomial errors and Tukey comparisons between species to determine whether significant differences occurred. The former were conducted using the glm.nb function from the MASS library, and the Tukey comparisons on the resulting glm.nb object were made using the glht function from the multcomp library. All analyses were conducted in the statistical software package R (R Core Team 2014).

RESULTS

EXPERIMENT 1

The prevalence of *Bd* in *Bufo spinosus* tadpoles at the beginning of the experiment was 0% according to qPCR analyses. The prevalence of infection in *B. spinosus* at the end of the experiment differed significantly among the 4 treatments (χ^2 = 38.23, df = 3, p < 0.001; Table 1). In the presence of *Ao* larvae, the prevalence of infection in *B. spinosus* was about 50%, while in the absence of *Ao* larvae, it was less than 7%. The fact that infection occurred suggests that infection can occur and persist via indirect transmission from zoospores in the water and is not initially reliant on direct contact with an infected host. Our model of infection probability was simplified to leave the presence/absence of *Ao* larvae as the only significant predictor of likelihood of infection (Table 2). Using a binomial test, we found no significant difference in the proportion of infection of *B. spinosus* larvae kept at low (2/25) and high density (3/50) in the absence of Ao (χ^2 < 0.001, df = 1, p = 1.000), indicating that regardless of the density of *B. spinosus*, infection did not become well established in the absence of *Ao*. Because of the very low number of infected animals in each of these 2

treatments, it was not useful to compare infection burden between treatments.

Table 1. Prevalence of infection in *Bufo spinosus* larvae in each of the 4 experimental treatments in Expt 1 (χ^2 = 38.23, df = 3, p < 0.001).

	Infected	Uninfected
Bs HD, - *Ao*	3	49
Bs HD, + *Ao*	28	26
Bs LD, - *Ao*	2	24
Bs LD, + *Ao*	17	19

_Bs_ HD/LD indicates high/low density of *B. spinosus* larvae and +/− Ao indicates presence/absence of *Alytes obstetricans* larvae

Table 2. Minimal adequate model of infection prevalence in *Bufo spinosus* in Expt 1.

	Coefficient	Transformed coefficient	SE	z	p
- *Ao*	-2.6810	0.06	0.4623	-5.80	<0.001
+ *Ao*	2.6810	0.5	0.5081	5.277	<0.001

df = 166, negative logLikelihood = 80.956
+/− _Ao_ indicates presence/absence of *Alytes obstetricans* larvae

The model of infection intensity contained both density of hosts and the presence/absence of *Ao* as factors affecting infection intensity in *B. spinosus* tadpoles. The output for this model is presented in Table 3. The model fit could not be significantly improved by the backwards stepwise regression process, meaning that the best-fitting model was obtained when both terms were left in the model.

Table 3. Minimum adequate model of infection intensity in *Bufo spinosus* larvae when housed at different densities with (+) and without (−) *Alytes obstetricans* (Ao) larvae in Expt 1.

	Coefficient	SE	z	p
Bs HD, - *Ao*	1.727	0.665	1.763	0.078
Bs HD, + *Ao*	2.303	0.930	2.478	0.012
Bs LD, - *Ao*	-21.475	3048.011	-0.007	0.994
Bs LD, + *Ao*	3.555	1.036	3.430	0.001

df= 164, negative logLikelihood = 242.625
***Bs* HD/LD indicates high/low density of *B. spinosus* larvae**

Tukey's HSD suggested that *B. spinosus* larvae housed at high density in the presence of *Ao* larvae had a significantly higher infection burden than those at high density without *Ao* larvae, and that *B. spinosus* larvae held at low density in the presence of *Ao* larvae had a higher infection intensity than *B. spinosus* larvae at high density in the absence of *Ao* (Table 4).

Table 4. Tukey's HSD test showing differences in infection intensity among 4 treatment levels in Expt 1.

	Estimate	SE	z	p
Bs HD + *Ao* / *Bs* HD - *Ao*	2.303	0.925	2.478	0.048
Bs HD + *Ao* / *Bs* LD + *Ao*	1.251	1.026	1.220	0.565
Bs HD + *Ao* / *Bs* LD - *Ao*	23.779	3048.011	0.008	1.000
Bs HD - *Ao* / *Bs* LD + *Ao*	-3.555	1.036	-3.430	0.002
Bs HD - *Ao* / *Bs* LD - *Ao*	21.475	3048.01	0.007	1.000
Bs LD + *Ao* / *Bs* LD - *Ao*	25.030	3048.011	0.008	1.000

Bs HD/LD indicates high/low density of *Bufo spinosus* larvae and +/− Ao indicates presence/absence of *Alytes obstetricans* larvae.

EXPERIMENT 2

When co-housed with *Ao* larvae, the 6 species showed no significant difference from one another in their probability of becoming infected (Fig. 1; Fisher's exact test: $p = 0.072$).

Four of the 6 co-housed species had significantly higher infection intensity in the presence of *Ao* than when housed only with conspecifics (*B. spinosus*: $t = 3.097$, df = 12, $p = 0.009$; *B. calamita*: $t = 4.705$, df = 9, $p = 0.001$; *Hyla molleri*: $t = 3.399$, df = 13, $p = 0.0475$; *Salamandra salamandra*: $t = 0.377$, df = 14, $p = 0.741$; *Pelophylax perezi*: $t = 4.582$ df = 14, $p < 0.001$; *Rana iberica*: $t = 2.037$, df = 12, $p = 0.064$; Fig. 1). Species was a significant predictor of infection intensity in our negative binomial glm ($F = 9.712$, df = 5, $p < 0.001$), and Tukey's HSD tests highlighted significant differences in the infections between those species (Table 5). *R. iberica* had a significantly lower infection level than *B. spinosus*, *B. calamita* and *H. molleri*. *S. salamandra* had a lower infection intensity than the latter 3 species plus *P. perezi*. *H. molleri* had a significantly higher infection intensity than *P. perezi*.

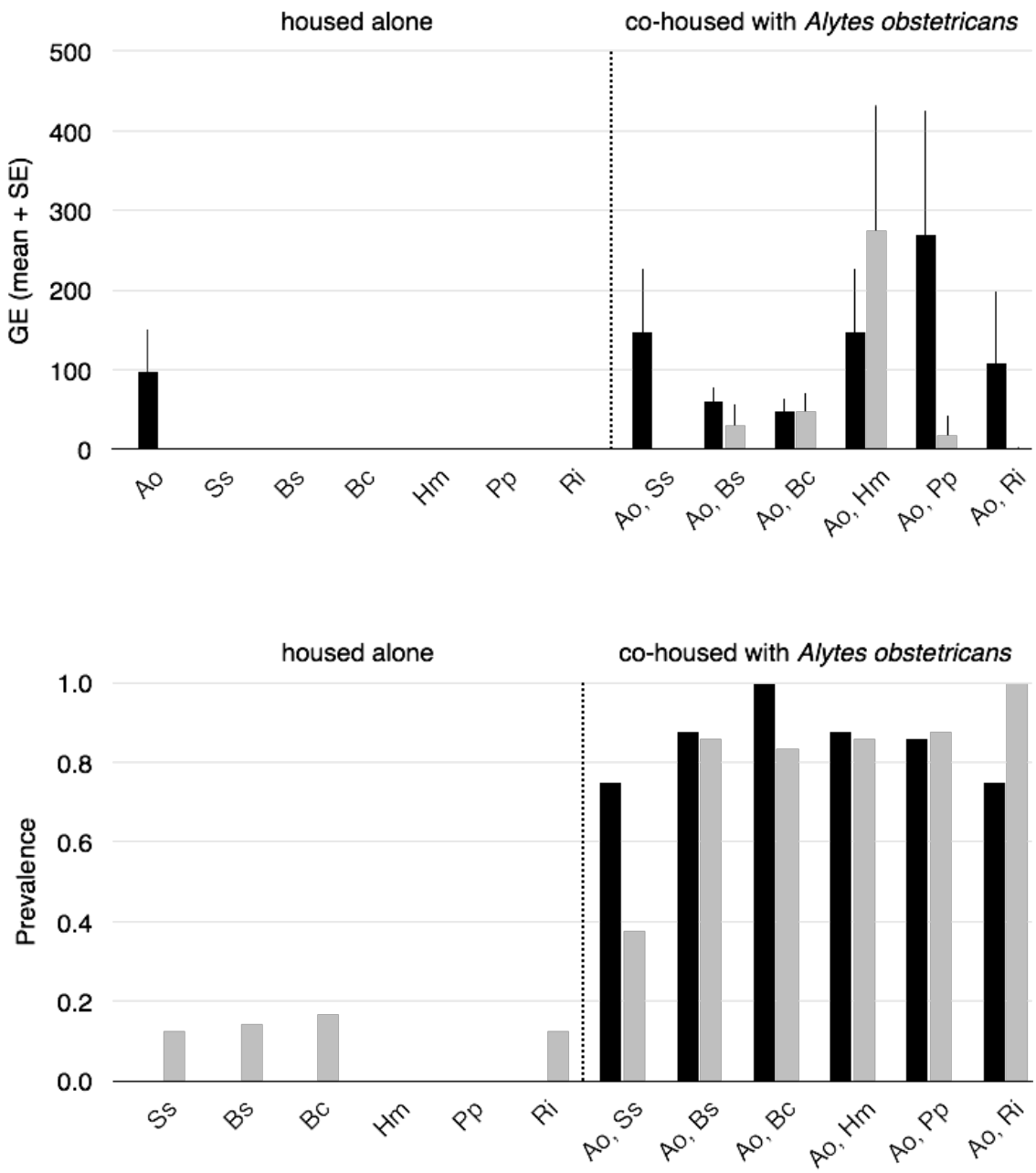

Figure 1. Infection intensity (mean + SE) and prevalence for the studied species when housed alone (left side) and when co-housed with *Alytes obstetricans* (Ao, right side) in Expt 2. Black bars are for *Ao*, gray bars are for the other species: *Salamandra salamandra* (*Ss*), *Bufo spinosus* (*Bs*), *Bufo calamita* (*Bc*), *Hyla molleri* (*Hm*), *Pelophylax perezi* (*Pp*) and *Rana iberica* (*Ri*)

There were no significant differences in the proportion of individuals infected or the infection intensity in *Ao* larvae when co-housed with different species (Fig. 1; Fisher's exact test, p = 0.796). This result is most likely because, by the end of the experiment, most *Ao* larvae were fairly heavily infected (Fig. 1).

EXPERIMENT 3

Individuals of other species co-housed with *Ao* did become infected, suggesting that *Ao* can transmit infection to other species. A Fisher's exact test on the species co-housed with *Ao* suggested no significant difference among those species in their probability of infection ($p = 0.2126$; Fig. 2). Significant differences in infection intensity were present among those species (Fig. 2; $F = 4.9807$, $df = 4$, $p < 0.001$). *P. perezi* had a significantly higher in fection intensity than *H. molleri*, *Triturus marmoratus* and *S. salamandra*. *Ichthyosaura alpestris* had heavier infections than *H. molleri* and *T. marmoratus* (Table 6).

The prevalence of infection in *Ao* varied significantly depending on whether they were housed alone or with the larvae of other species (Fig. 2; Fisher's exact test, $p = 0.0036$). *Ao* larvae experienced differences in infection intensity depending upon the species with which they were cohoused ($F = 5.068$, $df = 5$, $p < 0.001$). *Ao* housed alone had significantly lower infection burdens than when housed with any species aside from I. *alpestris*, when the infection intensity in *Ao* did not differ from when housed alone. *Ao* larvae housed with *H. molleri* had significantly higher infections than *Ao* larvae housed with *I. alpestris* (Table 7).

Table 5. Pairwise comparisons of infection intensity between the 6 species co-housed with Alytes *obstetricans* larvae in Expt 2.

	B. calamita		*H. molleri*		*P. perezi*		*R. iberica*		*S. salamandra*	
	z	p	z	p	z	p	z	p	z	p
B. spinosus	0.463	0.997	2.538	0.112	0.624	0.989	↑,3.077	0.025	↑,4.250	<0.001
B. calamita	-	-	1.855	0.428	1.042	0.903	↑,3.280	0.013	↑,4.368	<0.001
H. molleri	-	-	-	-	↑,3.243	0.014	↑,4.368	<0.001	↑,3.243	<0.001
P. perezi	-	-	-	-	-	-	2.590	0.099	↑,3.796	<0.002
R. iberica	-	-	-	-	-	-	-	-	1.147	0.860

Arrows indicate the relative infection intensity of the species named in the row compared to the species named in the column.

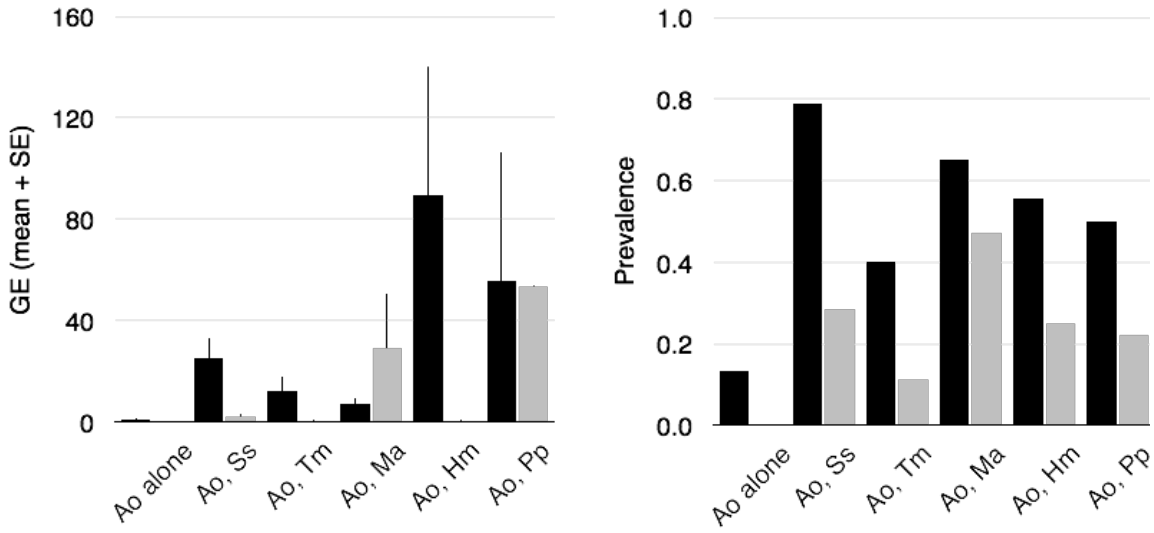

Figure 2. Infection intensity (GE: *Bd* zoospore genomic equivalent, mean + SE) and prevalence of species co-housed with *Alytes obstetricans* (Ao, black bars) larvae in Expt 3. Gray bars represent the other species: *Salamandra salamandra* (Ss), *Triturus marmoratus* (Tm), *Ichthyosaura alpestris* (Ia), *Hyla molleri* (Hm) and *Pelophylax perezi* (Pp).

Table 6. Tukey's HSD tests of infection intensity in species co-housed with *Alytes obstetricans* larvae in Expt 3.

	I. alpestris		*P. perezi*		*S. salamandra*		*T. marmoratus*	
	z	p	z	p	z	p	z	p
H. molleri	↓,2.890	0.032	↓,3.445	0.005	0.773	0.940	0.365	0.987
I. alpestris	-	-	0.500	0.987	2.200	0.180	↑,3.144	0.015
P. perezi	-	-	-	-	↑,2.757	0.046	↑,3.680	<0.002
S. salamandra	-	-	-	-	-	-	1.115	0.798

Arrows indicatethe relative infection intensity of the species named in the row compared to the species named in the column.

DISCUSSION

Within an assemblage of hosts, it is difficult to predict whether a parasite will become established, will spread or will cause disease because of heterogeneity in host response within a community. Our study shows that all species of the Peñalara Massif are susceptible to *Bd* infection, and that their levels of susceptibility vary greatly from one to another. As a result, different species are likely to play different roles in the infection dynamics

within the system. Of particular note, our data suggest that the larvae of 1 species, *Ao*, could contribute a disproportionate amount to the spread of infection and, in so doing, may act as an amplification host. By carrying severe infections, it may cause co-housed species to experience elevated levels of infection, and by transmitting directly to other species, overwintering *Ao* larvae may play the role of amplification host within this host community.

Table 7. Tukey's HSD tests between *Alytes obstetricans* larvae (*Ao*) co-housed with different species in Expt 3.

	H. molleri		*I. alpestris*		*P. perezi*		*S. salamandra*		*T. marmoratus*	
	z	p	z	p	z	p	z	p	z	p
Control (*Ao* alone)	↓,5.201	0.001	2.483	0.128	↓,4.774	0.001	↓,3.827	0.001	↓,2.871	0.047
H. molleri	-	-	↑,3.109	0.023	0.594	0.991	1.576	0.613	2.313	0.188
I. alpestris	-	-	-	-	2.585	0.100	1.583	0.638	0.591	0.992
P. perezi	-	-	-	-	-	-	1.106	0.912	1.804	0.462
S. salamandra	-	-	-	-	-	-	-	-	0.843	0.959

Arrows indicate the relative infection intensity of the species named in the row compared to the species named in the column.

The ability of overwintering amphibian larvae to act as infection reservoirs is well established (Brunner *et al.* 2004, Narayan *et al.* 2014, Medina *et al.* 2015), yet there is little empirical evidence to suggest that they can increase levels of infection within a host assemblage. Combined, the results of our experiments suggest that *Ao* larvae are able to increase infection prevalence and intensity in a number of cohoused species by directly transmitting infection to them. Further, the ability of *Ao* to act as an amplification host appears to be independent of the overall density of larvae around it, as highlighted in Expt 1. In this experiment, the density of the co-housed focal species *Bufo spinosus* did not affect its likelihood of becoming infected, which remained close to 0 in the absence of *Ao*. In contrast, *B. spinosus* larvae held at low density in the presence of *Ao* larvae had a higher infection intensity than *B. spinosus* larvae at high density in the absence of *Ao*, suggesting that the presence of a single *Ao* larva resulted in a significant increase in infection probability and intensity regardless of overall host density. The fact that the presence of *Ao* is strongly associated with infection in other species, regardless of the overall density of hosts, suggests that even post-decline, when the overall density of hosts is reduced, infection may still be maintained and spread if *Ao* larvae remain present in the community.

We considered what characteristics would predispose *Ao* to act as reservoirs or disseminators of infections in the shorter term. One possible morphological feature that would lend itself to a species harbouring and transmitting high levels of infection is its large oral disc. In *Ao*, this feature is unusually large, and includes numerous rows of large denticles with a high concentration of keratin. Therefore, it has a greater area available to be infected by the pathogen (Berger *et al.* 1998; but see Searle *et al.* 2011, who reported that smaller species, such as *Anaxyrus boreas*, presented the highest infection loads). This potential mechanism could be explored further using techniques to track infection prevalence and infection intensity in different body parts, and it highlights the importance of understanding species' biology when considering their roles in transmission of infection within a community of hosts.

Efforts to better understand how and when transmission of infection will take place rely greatly on accurate information about mechanisms and modes of transmission. Within this host-pathogen system, it is generally assumed that, given the low motility of *Bd* zoospores (Moss *et al.* 2008, Lam *et al.* 2011) and the tendency of amphibians to cluster at high densities in suitable conditions (Duellman & Trueb 1994), direct host contact may be the most common method of infection transmission. However, the data from Expt 1 suggest that initial infection can and does occur as a result of exposure to infected lake water by means of zoospores present in the lake. This finding supports previous research in demonstrating that transmission of infection does not necessarily require direct contact between the tadpoles (Rachowicz & Briggs 2007) and can help to inform future efforts to understand transmission events within this host-parasite system.

A great deal of variation in host susceptibility to *Bd* infection was observed within our Expts 2 and 3. Although the majority of species had an increased probability of infection and infection intensity in the presence of *Ao* larvae, there was a considerable degree of variation among species as to how prevalent or severe those infections became. These differences reflect how the transmission dynamics within a community may differ depending upon its constituent species. This observation makes it difficult to predict how host communities will respond to the introduction of *Bd*. Additionally, there was little consistency in how the studied species responded to *Bd* introduction levels among the performed experiments (for example, *Hyla molleri* and *Pelophylax perezi* in Expts 2 and 3).

Infection levels varied not only in those species co-housed with *Ao*, but also in *Ao* larvae depending upon the species with which they were housed. Cohoused *Ao* generally suffered more frequent and heavier infections than those housed alone, but those with *Ichthyosaura alpestris* did not, having significantly lower infections than *Ao* housed with *H. molleri* larvae. While it is currently difficult to determine the mechanism behind these differences, the end result is that, even for a host capable of carrying heavy infection burdens, competition with other larvae, or the ability for infection to be transmitted both to and from other species in the assemblage, may, at times, be important for the maintenance of infection. These inconsistencies and inter-species differences suggest that the outcome of *Bd* exposure is highly context dependent and may differ greatly depending upon the source of infection and the environment in which the larvae develop, illustrating how important it is to carefully consider the generalities of research into the transmission within any host-parasite system.

Rachowicz & Briggs (2007) showed that under laboratory or field conditions, there is a clear influence of the density of infected individuals on the rates of *Bd* transmission. The density of both host and pathogen are fundamental parameters in the transmission of infectious disease. Although the experimental numbers of tadpoles in our study were similar to those used in the study mentioned above, we did not find a significant effect of density of tadpoles in the variation of *Bd* infection intensity. Our experimental design was such that comparisons of species cohoused with and without *Ao* varied not only in species composition, but also in the density of animals in the experimental treatments. Accounting for both density and species composition would be the ideal approach to take, but the practicalities involved with conducting such experiments prevented these dual comparisons. Regardless of these different densities, the main findings of our experiments remain unchanged. We conclude that *Ao* presence/absence is a greater predictor of infection than overall density of tadpoles (Expt 1), that species co-housed with *Ao* differ in their response to parasite exposure (Expts 2 and 3), that *Ao* varies in its infection levels depending on the species with which it is housed and that *Ao* can directly infect other species (Expt 3).

To add more complexity to the overall findings, competition and stress between 2 host species may account for some of the observed patterns. Additional experiments with the target host at different densities and addition of non-target and non-*Ao* hosts would be needed to test

whether additional host species simply cause competitive stress and thus lead to increased infection.

Identifying the roles that different species or life stages play in the transmission, prevalence and intensity of infection is crucial to better understand the persistence and spread of infection within a host-pathogen system. Knowledge related to which species are more tolerant and more susceptible to infection could allow designers of mitigation efforts to focus on reducing the levels of infection in a host. In the case of our study system, this might be accomplished by aiming to reduce the amount of infection in potential 'amplifier' hosts. Considering the species composition of a particular host community is essential in efforts to understand the spread of infection, risk of disease emergence and, ultimately, in managing systems to minimize any negative effects of pathogens on biodiversity.

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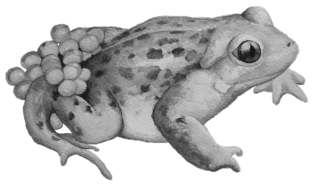

CAPÍTULO 8

DEMOGRAFÍA DEL SAPO COMÚN TRAS LA DESAPARICIÓN LOCAL DEL SAPO PARTERO COMÚN

DEMOGRAPHY OF COMMON TOADS AFTER LOCAL EXTIRPATION OF CO-OCCURRING MIDWIFE TOADS

DEMOGRAFÍA DEL SAPO COMÚN TRAS LA DESAPARICIÓN LOCAL DEL SAPO PARTERO COMÚN

RESUMEN

La estima de parámetros demográficos como la supervivencia o reclutamiento provee información sobre el estado y la trayectoria de poblaciones, pero entender el contexto que afecta a estos parámetros, incluyendo tanto factores bióticos como abióticos, es particularmente importante para su gestión y conservación. En un parque nacional de elevada altitud en el centro de España, el sapo común (*Bufo spinosus*) está tomando ventaja, aparentemente, tras la reciente desaparición del sapo partero común (*Alytes obstetricans*), pues la colonización de nuevos puntos de reproducción es evidente. Dentro de este escenario esperamos que los parámetros demográficos del sapo común se vean afectados favorablemente por la eliminación de competencia. Sin embargo, encontramos que la tasa de crecimiento de la población fue negativa en 4 de los 5 años estudiados en los lugares de colonización antigua; la probabilidad de supervivencia, tanto en los lugares de colonización antigua como en los de reciente colonización, fue más baja que la observada para otros sapos viviendo a elevada altitud y la tasa de reclutamiento no fue suficiente para compensar la probabilidad de supervivencia y mantener una tendencia de crecimiento positivo en la población en ninguno de los puntos de reproducción. Evaluamos la contribución de variables ambientales y el efecto de la quitridiomicosis al contexto que puede estar limitando el éxito del sapo común a la hora de utilizar el nicho que ha dejado vacante el sapo partero común.

DEMOGRAPHY OF COMMON TOADS AFTER LOCAL EXTIRPATION OF CO-OCCURRING MIDWIFE TOADS

ABSTRACT

Estimating demographic parameters like survival or recruitment provides insight into the state and trajectory of populations, but understanding the contexts influencing those parameters, including both biotic and abiotic factors, is particularly important for management and conservation. At a high elevation national park in Central Spain, common toads (*Bufo spinosus*) are apparently taking advantage of the near-extirpation of the midwife toad (*Alytes obstetricans*), as colonization into new breeding ponds is evident. Within this scenario, we expected demographic parameters of common toad populations to be affected favorably by the putative release from competition. However, we found the population growth rate was negative in 4 of 5 years at the long-standing population; survival probability at the long-standing population and newly-colonised breeding ponds was lower than reported for other toads living at high elevations and the probability of recruitment was inadequate to compensate for the survival rate in maintaining a positive trajectory for either of the breeding ponds. We assessed weather covariates and disease for their contribution to the context that may be limiting the common toad's successful use of the niche vacated by the midwife toad.

INTRODUCTION

Biotic interactions among species range from complete exclusion of one species by another, to co-occurrence in the same habitat where interaction may affect demographic parameters in one or both species, (e.g., survival as indexed by decreased body mass at metamorphosis and increased larval period length in *Rana areolata* compared to co-occurring species, Parris & Semlitsch 1998), to niche partitioning (e.g., differential use of habitat, Indermaur *et al.* 2009). When one species is extirpated and leaves a co-occurring, ecologically similar species in its place, population growth and persistence by the remaining species may be due to a release from competition and adaptations that allow it to better cope with the changes that contributed to the other species' extirpation (e.g., emerging disease, changing climate). Under these conditions, we expect the remaining species to colonize breeding ponds vacated by the extirpated species and to exhibit increased population growth prior to reaching the breeding pond's carrying capacity. However, short-term success, facilitated by the extirpation of the other species, may not translate into long-term persistence. Accounting for biological associations, as well as abiotic factors, can provide information to better understand the long-term trajectory of populations and the consequences of location.

The midwife toad (*Alytes obstetricans*) and the common toad (*Bufo spinosus*) are found in Guadarrama National Park (GNP), in Central Spain. The presence of midwife toad tadpoles reduces mass at metamorphosis, growth rate, and survival of common toad tadpoles in the laboratory (Richter-Boix *et al.* 2007). In the field, Bosch & Rincón (2008) reported an inverse relationship between the number of common toad clutches laid and the number of midwife toad tadpoles present at a breeding pond. In the laboratory Bosch & Rincón (2008) tested whether or not midwife toad tadpoles consumed common toad eggs, as a mechanism behind this relationship, and found that while common toad eggs were not consumed by midwife toad tadpoles, common toads avoided laying eggs in tanks where overwintering midwife toad tadpoles were present. Therefore, the presence of midwife toads appears to exclude or suppress common toads.

Between 1997 and 2002, midwife toads declined dramatically in GNP as a result of chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Bosch *et al.* 2001). Common toads are not compromised as severely by *Bd* as midwife toads, likely because they exhibit traits that theoretically reduce the impact of *Bd* (e.g.,

Lips *et al.* 2003). However, *Bd* generally incurs a cost for common toad tadpoles in terms of growth and can cause larval mortality before or soon after metamorphosis (Bosch & Martínez-Solano 2006; Garner *et al.* 2009). Between 1999 and 2004, common toads, historically present only in the largest pond (Laguna Grande) in higher altitude areas of GNP, colonized 5 ponds vacated by the extirpation of midwife toads (Bosch & Rincón 2008). Midwife toads returned to Laguna Grande in 2005, when the non-native brook trout (*Salvelinus fontinalis)* was finally extirpated, but its recent presence can be considered anecdotal since less than 20 tadpoles were counted since then.

While these observations suggest that successful colonizations are taking place in the wake of the extirpation of midwife toads, an examination of the populations at the original and more recently colonized breeding ponds may provide insights into the demography of colonization. We expected common toads at colonized breeding ponds to be thriving because of a lack of competition, lower density, and an apparent increased ability to co-exist with the disease (*Bd*). We also expected a high population growth rate (lambda) at colonized breeding ponds.

The long-term persistence of populations, both established and colonial, depends on a variety of factors. Weather (an abiotic factor) can be extreme at high elevation breeding ponds, negating even the most fecund reproductive effort (e.g., Scherer *et al.* 2008). Competitive interactions with other species (biotic factors) can also affect long-term persistence. In addition to more obvious effects of competition among co-occurring species, interactions among classes of organisms can affect persistence. The effect of the amphibian chytrid fungus appears to be reduced in common toads, but based on disease dynamics and density dependent behaviors of both the toads and the fungus (*sensu* Briggs *et al.* 2010), this relationship has the potential to change as density of common toads increases. Characteristics of the colonizer (other biotic factors) such as foraging ability are also potentially influential in the long-term persistence of a population. For common toads at GNP, the changes wrought by the extirpation of the midwife toad provided an opportunity, but their long-term persistence depends on their ability to exploit the niche and respond to challenges (i.e., disease, weather).

To better understand the demography of colonization by the common toad in GNP, we estimated demographic parameters (survival probability, recruitment rate, and population growth rate) at a recently

colonized breeding pond (Laguna Chica) and at the pond where common toads have been present for at least two decades (Laguna Grande) (Fig. 1). We also evaluated hypothesized relationships between demographic rates and weather conditions. The majority of the covariates that we evaluated describe conditions during the time of year when anurans, particularly at high elevations, are most vulnerable (e.g., Scherer *et al.* 2008). For example, breeding and preparing to hibernate are particularly critical (Reading 2007). We relate model selection results to data from previous research at these breeding ponds and build a plausible scenario to explain the changes in the distribution of common toads in higher altitude areas of GNP.

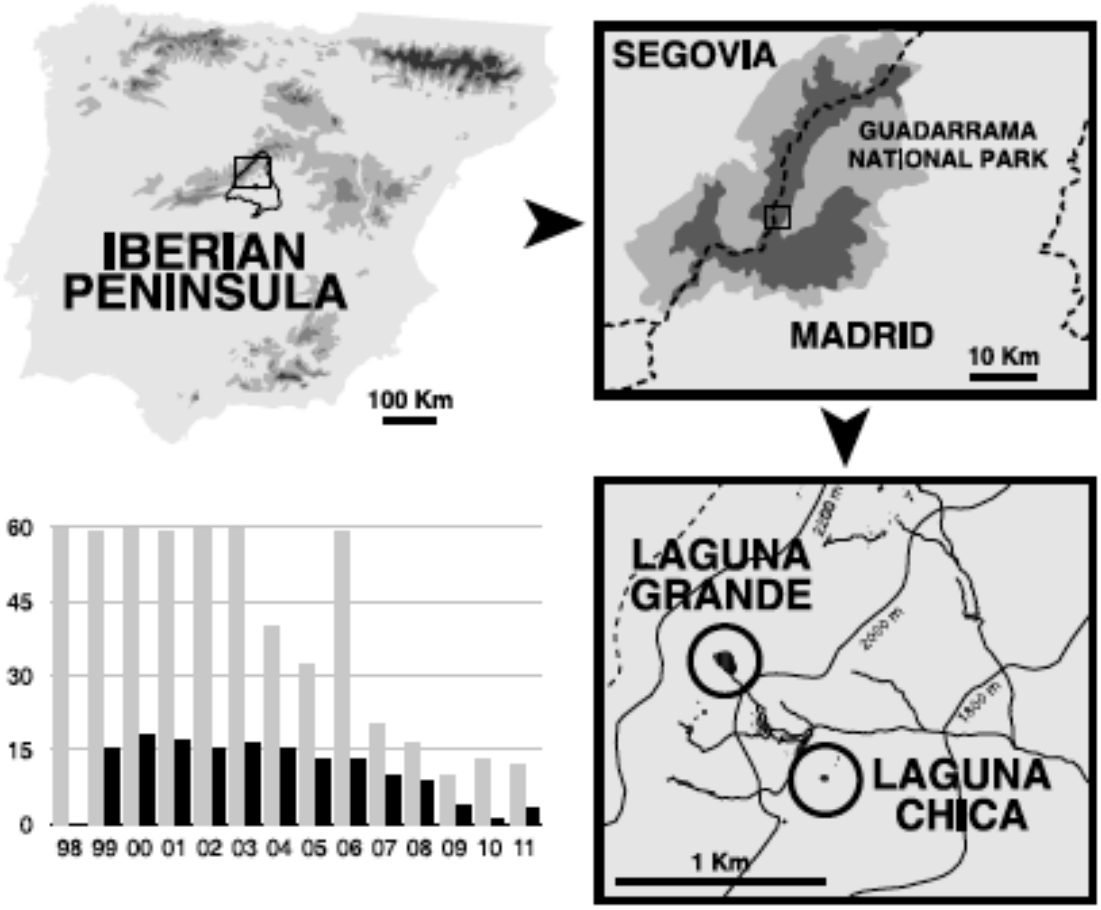

Figure 1. (Top and bottom right) Location of the studied breeding ponds Laguna Grande and Laguna Chica, within Guadarrama National Park in the Iberian Peninsula. (Bottom left) Temporal changes in the number of *B. spinosus* clutches in these ponds from 1998 to 2011; Laguna Grande: grey bars; Laguna Chica: black bars.

METHODS AND MATERIALS

STUDY SITE

Laguna Grande (elevation 2.018 m, surface 5.452 m^2, maximum depth 2.7 m) and Laguna Chica (elevation 1.956 m, surface 739 m^2, maximum depth 0.7 m) are two of the largest ponds in GNP, Sierra de Guadarrama, central Spain, near Madrid (41°N, 4°W; Fig. 1). The park is a protected, alpine habitat (1.800-2.430 m) and ponds are above the tree line with little vegetation around the shore (Toro & Granados 1999). Both sites are above the tree line and isolated, so that they are the only water bodies in the same hydrographic basin where *B. spinosus* breeds (Bosch & Rincón, 2008).

DATA COLLECTION

Routine yearly censuses were carried out in higher altitude areas of GNP since 1999, and common toad clutches and over-wintering midwife toad tadpoles were counted individually whenever possible. Additionally, data on the distribution and reproduction of amphibian species in each of the 250 cataloged ponds in the area since 1982 is available. We conducted multiple nighttime capture sessions to collect data during the breeding season (April - June) from populations of common toads at Laguna Grande and Laguna Chica from 2006 to 2011. We completed 2-7 sessions each breeding season (Table 1). During each session, we searched the entire shoreline and all animals were captured by dip net. We recorded sex, mass, snout-vent-length (SVL) and PIT (passive integrated transponder) tag number for each captured toad. If animals did not already have a tag, a new tag was inserted subcutaneously on the dorsal side. Toads were released at the point of capture immediately after data were collected. In 2009, a sub sample of adults at Laguna Grande and Laguna Chica were tested for *Bd* using standard field techniques and quantitative molecular methods (Hyatt *et al.* 2007) resulting in estimates for *Bd* load for each individual at each breeding pond.

Table 1. Number of capture sessions and results from disease (*Bd*) sampling.

Breeding pond	Capture session					Prevalence (# Positive/# Tested)	Mean zoospore count
	2006	2007	2008	2009	2010	2009	2009
Laguna Chica	2	9	8	7	3	0.35 (16/46)	115.04
Laguna Grande	16	14	12	9	21	0.42 (50/118)	1,556.99

ANALYSIS

We used the Pradel model (Pradel 1996) for data collected under Pollock's robust design (Pollock 1982) in Program MARK (White & Burnham 1999) to analyze the capture-recapture data for male toads only. Data collected under the robust design are characterized by multiple capture sessions within a year (each capture session is referred to as a secondary occasion). The collection of secondary occasions withinin each year is referred to as a primary period (Pollock 1982, Kendall *et a*l. 1997) thus primary periods were approximately 2 months with 10 months in between. The model assumes that the time between secondary occasions is sufficiently short that individuals are not added to or lost from the population (i.e., the closure assumption). The amount of time between primary periods, on the other hand, is long enough for gains and losses to occur (e.g., recruitment or mortality).

Because no gains or losses are assumed to occur across secondary occasions within a primary period, the only process being modeled is the capture process. On each occasion, an individual in the study area is either captured for the first time within a primary period, recaptured after being captured earlier within a primary period or not captured. The probabilities of these outcomes are represented as p (the probability of first capture) and c (the probability of recapture), while failing to capture an individual is represented as $1 - p$ or $1 - c$. We used closed population models (Otis *et al.* 1978) to model p and c for secondary occasions within each primary period (Williams *et al.* 2002).

While the capture-recapture data within primary periods are used to estimate p, c, and N, the data across primary periods can be used to estimate a variety of demographic parameters (Pradel 1996, Williams *et al.* 2002). We used the f-parameterization of the Pradel (1996) model to estimate apparent survival probability and recruitment rate between primary periods. Apparent survival probability (hereafter, survival probability, Φ_t), is the probability of an animal surviving and remaining in the study area between primary periods t and $t + 1$. The presence of temporary emigration (i.e., absence from the breeding pond in one or more years, but return to the breeding pond in subsequent years) can bias estimates of survival probability negatively (Kendall *et al.* 1997, Converse *et al.* 2009). We assume that estimates of survival probability from our analyses will be minimally biased because male toads in similar environments are seldom absent from breeding ponds (Muths *et al.* 2006)

and analyses of the capture recapture data from Laguna Grande suggests that males do not temporarily emigrate (Muths *et al.* 2013). We define recruitment rate, f_t as the per capita number of individuals added to the breeding population between primary periods t and $t + 1$. Within Program MARK, we used the estimates of Φ_t and f_t to derive estimates of the finite population growth rate, λ_t, and associated standard errors using the equation $\lambda_t = \Phi_t + f_t$. The finite population growth rate can also be written as the ratio between population sizes at time $t + 1$ and t ($\lambda_t = N_{t+1}/N_t$) where N represents population size. If $\lambda_t > 1$, the number of individuals in the population increased between t and t+1, whereas $\lambda_t < 1$ indicates a declining population (Williams *et al.* 2002).

Our interests were first to estimate Φ_t, f_t, and λ_t, and then to examine differences in those parameters between the populations at Laguna Chica and Laguna Grande. Second, we evaluated hypothesized causes for the temporal variation in the parameters. We hypothesized that survival and recruitment are higher with "good" weather, as represented by several covariates that we discuss below. Recruitment rate is partly dependent on conditions at the time of egg laying or metamorphosis and it takes approximately 2-4 years for common toads to reach breeding age (Reading 1991). Therefore, for weather covariates hypothesized to be correlated with recruitment rate, we used the value for that covariate 2, 3 and 4 years previous to the year of recruitment (i.e., we lagged the effects of the weather covariates by 2, 3 and 4 years). Models that included 4-year lags had greater support; therefore, we excluded models with 2- and 3- year lags.

In capture-recapture analyses, models of Φ_t, f_t, and p_t, are not fit to the data separately. A model for each parameter is combined with a model for the other parameters prior to fitting them to the data. We first developed a set of *a priori* hypotheses for capture probability and recapture probability for each population. In preliminary models, we found no evidence for heterogeneity between capture probability, p, and recapture probability, c; therefore we fixed $p = c$ for all subsequent models.

For each population, we assessed the following hypotheses regarding temporal variation in capture probability within each primary period (*sensu* Muths *et al.* 2011): i) capture probability will vary across all secondary occasions (where secondary occasion is a categorical variable), ii) capture probability will increase to a peak early in each primary period, then decline in later secondary occasions (a quadratic function), and iii) capture probability will decrease across secondary occasions (a linear trend). We

also used weather data (*sensu* Scherer *et al.* 2008) from the days of the secondary occasions to determine if capture probability was correlated with median air temperature, average wind speed, the amount of precipitation, barometric pressure or a combination of median air temperature and average wind speed. We obtained climatic data from the nearest meteorological station located at a similar altitude (Puerto de Navacerrada, 1.890m, 40º46'50'' N, 4º00'37''W), 5.4 km away from the study area. We quantified the support for each model with ΔAICc values, Δ_i, and Akaike weights, w_i (Burnham & Anderson, 2002) and used all models of p_t with ΔAICc < 6 in subsequent stages of modeling.

After identifying the most strongly supported model or models of capture probability, p, we specified models for f_t. We developed 8 hypotheses and associated mathematical models for f_t (Table 2A). The set of models includes effects of primary period on recruitment rate (where primary period is modeled as a categorical variable) and effects of six weather covariates, as well as a model of no variation in recruitment rate across primary periods. Hypotheses were based on our understanding of toad biology and observations by J.B. at these sites.

The choice of weather covariates reflect our prediction that conditions the winter before tadpoles metamorphosed, and the late-summer and winter after metamorphosis are most critical to the survival of metamorphs and their subsequent recruitment into the adult population. We presume that higher precipitation in the winter before metamorphosis leads to wetter conditions and higher productivity in the year of metamorphosis; wetter and warmer conditions in the September of the year in which metamorphosis takes place delay or prevent desiccation of breeding ponds, promoting growth of metamorphosed individuals prior to their first winter; and that warmer winters after metamorphosis facilitate survival. We used all models of f_t with ΔAICc < 6 in subsequent models of Φ_t (Table 2B).

Finally, we combined the highest ranked models of p_t and f_t with every model of Φ_t, fit the models to the data and evaluated them using Δ_i and w_i to identify the models with the most support (Burnham & Anderson 2002). We hypothesized that cold winters, winters with variable temperatures, and hot summers would affect survival probability of common toads negatively (Scherer *et al.* 2008, Table 2B). We used model-averaging (Burnham & Anderson 2002) to derive estimates of Φ_t, f_t, and λ_t. To evaluate the importance of weather covariates, we examined the 95%

confidence intervals of the estimates of regression coefficients. If confidence intervals do not include 0, we inferred a stronger association between the covariate and demographic parameter.

We used the results from *Bd* testing in 2009 to test the hypothesis that individuals with high *Bd* loads had lower survival probabilities than individuals with low *Bd* loads. For this analysis, we only used the capture-recapture data for males that were tested (n = 118 at Laguna Grande; n = 46 at Laguna Chica). We pooled the capture-recapture data across secondary occasions such that each individual was recorded as captured or not for each primary period. Since sample sizes were small, we also pooled the data across the two populations. We analyzed the data using the CJS model (Lebreton *et al.* 1992) in Program MARK and compared models that included an effect of *Bd* on individual survival probabilities from 2009 to 2010 to models without the *Bd* effect.

RESULTS

We captured 462 male toads at Laguna Grande and 83 male toads at Laguna Chica between 2006 and 2010. The most strongly supported model of capture probability included variation among secondary occasions and primary periods. In both populations, the data provided no support for other models. Estimates of capture and recapture probabilities were small (≤ 0.25), but the probability of capturing an individual at least once in a primary period was high due to the large number of secondary occasions (estimates ranged from 0.46 to 0.90). With the exception of 2006, estimates of capture and recapture probabilities at Laguna Chica were higher (≤ 0.59).

The highest ranked models of recruitment rate, f_t, for the population at Laguna Grande suggested that recruitment is negatively associated with average daily air temperature (TMINWIN) during the winter after the tadpoles metamorphosed and also negatively associated with the amount of precipitation the winter before the tadpoles metamorphosed (PRECWIN) (Table 3). Both of the correlations were inconsistent with our hypotheses (Table 2A).

The model selection results for recruitment rate at Laguna Chica indicate support for multiple models. However, when they are combined with estimates of regression coefficients for the weather covariates, the strongest support is for a positive association between recruitment rate, f_t,

and the number of days with minimum temperature < 0 in the winter after tadpoles metamorphosed (NUMWIN). The 95% confidence interval for the estimated regression coefficient for NUMWIN is the only one that does not include 0. The positive association was not consistent with our hypothesis (Table 2A). Model-averaged estimates of recruitment rate in the two populations were similar in magnitude, though estimates at Laguna Chica were more variable among years (Fig. 2).

Table 2. Hypotheses for modeling recruitment rate and adult survival probability. Note that all covariates for recruitment rate are lagged by 4 year

Hypothesis	Model Name	Mathematical Model
A. Recruitment Rate		
1. Recruitment rate varies across primary periods	f(t)	$f_t=\beta_0+\beta_t(x_t)$
2. Recruitment rate does not vary between primary periods	f(.)	$f_t=\beta_0$
3. Recruitment rate is negatively correlated with the number of days with minimum temperatures in winter (1 Sept – 30 April) < 0° C during the first winter after metamorphosis	f(NUMWIN)	$f_t=\beta_0+\beta_1(NUMWIN_t)$
4. Recruitment rate is positively correlated with the average dailyminimum temperature in the winter after metamorphosis	f(TMINWIN)	$f_t=\beta_0+\beta_1(TMINWIN_t)$
5. Recruitment rate is positively correlated with the mean daily average temperature in September of the year of metamorphosis	f(SEPTEMP)	$f_t=\beta_0+\beta_1(SEPTEMP_t)$
6. Recruitment rate is positively correlated with the precipitation in September of the year of metamorphosis	f(SEPPREC)	$f_t=\beta_0+\beta_1(SEPPREC_t)$
7. Recruitment rate is negatively correlated with the Julian day on which the pond is free of ice in the spring in the year of metamorphosis	f(ICEFREE)	$f_t=\beta_0+\beta_1(ICEFREE_t)$
8. Recruitment rate is positively correlated with winter precipitation in the winter before metamorphosis	f(PRECWIN)	$f_t=\beta_0+\beta_1(PRECWIN_t)$
B. Survival Probability		
1. Survival probability varies across primary periods (primary period is modeled as a categorical variable)	Φ(t)	$\Phi_t=\beta_0+\beta_1(x_t)$
2. Survival probability does not vary	Φ(.)	$\Phi_t=\beta_0$
3. Survival probability is negatively correlated with the number of times the average daily temperature in winter (1 Sept to 30 April) changes from below freezing to above freezing	Φ(TEMPTRANS)	$\Phi_t=\beta_0+\beta_1(TEMPTRANS_t)$
4. Survival probability is negatively correlated with the number 7-day periods with average minimum daily temperature below -5° C	Φ(NUMPER)	$\Phi_t=\beta_0+\beta_1(NUMPER_t)$
5. Survival probability is negatively correlated with the number of days in winter with minimum temperature below 0° C	Φ(NUMPER)	$\Phi_t=\beta_0+\beta_1(NUMPER_t$
6. Survival probability is positively correlated with the average daily minimum temperature over the coldest 7-day period in winter	Φ(TMIN7AVE)	$\Phi_t=\beta_0+\beta_1(TMIN7AVE_t)$
7. Survival probability is negatively correlated with the highest daily maximum air temperature over summer (1 June to 30 Sept)	Φ(TMAXSUMR)	$\Phi_t=\beta_0+\beta_1(TMAXSUMR_t)$

Table 3. Model selection results shown for recruitment rate (A) and survival probability (B). Models with AIC_c Wt of < 0.1 are not shown.

	AIC_C	Deviance	ΔAIC_c	Model AIC_c Wt	Likelihood	Num.Par	
A. RECRUITMENT RATE							
LAGUNA GRANDE							
{phi(t), f(TMINWIN), p(t,t)=c(t,t), N}	3376.822	3193.081	0.000	0.620	1.000	87	
{phi(t), f(PRECWIN), p(t,t)=c(t,t), N}	3377.800	3194.060	0.979	0.380	0.613	87	
{phi(t), f(t), p(t,t)=c(t,t), N}	3445.157	3254.717	68.335	0.000	0.000	90	
{phi(t), f(.), p(t,t)=c(t,t), N}	3487.720	3306.207	110.899	0.000	0.000	86	
{phi(t), f(ICEFREE4), p(t,t)=c(t,t), N}	3490.222	3306.482	113.400	0.000	0.000	87	
{phi(t), f(NUMWIN) INIT, p(t,t)=c(t,t), N}	3558.886	3375.145	182.064	0.000	0.000	87	
LAGUNA CHICA							
{phi(t), f(NUMWIN), p(t,t)=c(t,t), N}	518.467		0.000	0.479	1.000	39	428.649
{phi(t), f(SEPTPREC4), p(t,t)=c(t,t), N}	519.455		0.987	0.293	0.610	40	426.983
{phi(t), f(SEPTTEMP4), p(t,t)=c(t,t), N}	521.082		2.615	0.130	0.271	39	431.264
{phi(t), f(TMINWIN), p(t,t)=c(t,t), N}	523.010		4.543	0.049	0.103	39	433.192
{phi(t), f(PRECWIN), p(t,t)=c(t,t), N}	523.882		5.414	0.032	0.067	39	434.063
{phi(t), f(t), p(t,t)=c(t,t), N}	526.544		8.076	0.008	0.018	43	425.990
{phi(t), f(ICEFREE4), p(t,t)=c(t,t), N}	527.863		9.396	0.004	0.009	40	435.392
{phi(t), f(.), p(t,t)=c(t,t), N}	528.121		9.654	0.004	0.008	39	438.303
B. SURVIVAL PROBABILITY							
LAGUNA GRANDE							
{phi(t), f(TMINWIN), p(t,t)=c(t,t), N}	3376.822	3193.081	0.000	0.620	1.000	87	
{phi(t), f(PRECWIN), p(t,t)=c(t,t), N}	3377.800	3194.060	0.979	0.380	0.613	87	
LAGUNA CHICA							
{phi(TEMPTRANS), f(SEPTPREC4), p(t,t)=c(t,t), N}	512.020		0.000	0.211	1.000	37	427.449
{phi(NUMWIN), f(SEPTPREC4), p(t,t)=c(t,t), N}	512.353		0.333	0.179	0.847	37	427.782
{phi(TMIN7AVE), f(SEPTPREC4), p(t,t)=c(t,t), N}	513.382		1.362	0.107	0.506	37	428.811

The model selection results provided no evidence for a correlation between survival probability and any of the weather covariates at Laguna Grande, though there was strong support for variation among years (Table 3). There was substantial uncertainty in model selection for survival

probability at Laguna Chica, but in the top three models, the estimated relationships between survival probability and weather covariates were consistent with our hypotheses. The probability of survival decreased as: a) the number of times the average daily minimum temperature changed from above freezing to below freezing and vice versa (TEMPRANS; Table 2B, 3); b) the number of days in winter with minimum temperature < 0 °C increased (NUMWIN; Table 2B, 5); and c) the average daily minimum temperature of the coldest 7-day period decreased (TMIN7AVE; Table 2B, 6). Estimates of survival probability were generally higher at Laguna Chica, particularly in 2007 (Fig. 2).

Of those toads tested for *Bd* in 2009, the proportion of individuals testing positive was similar (42% Laguna Grande and 35% Laguna Chica). Average prevalence was greater at Laguna Grande (Table 1) and almost twice as many samples had zoospore equivalents of > 20 at Laguna Grande (34%) relative to Laguna Chica (17%). However, we found no evidence that survival probability was lower for individuals with higher *Bd* loads or higher for individuals positive for *Bd* infection.

DISCUSSION

We do not have demographic estimates for common toads before midwife toads were extirpated, but toads at high elevations are typically long-lived (e.g., 10+ years; common toads, Cvetkovic *et al.* 2008, Zhang and Lu 2012; boreal toads, Muths & Nanjappa 2005). Longevity, plus a release from competition with midwife toads, led to our expectation of high survival probabilities at both breeding ponds. We further expected increased recruitment rate of adults at both ponds, because common toad tadpoles would have more resources in the absence of midwife toads. Estimates of population growth rate from the population at Laguna Chica suggest it is stable, but contrary to what we expected, estimates from the population at Laguna Grande indicate a decline (Fig.2). There was little support in the data for an effect of our selected weather covariates on the targeted parameters, due possibly to the scale at which covariates were measured or because patterns did not emerge over our short time frame. Mindful that our data cover a relatively small time period, there are a variety of plausible explanations for our results including a source - sink dynamic (*sensu* Pulliam 1988).

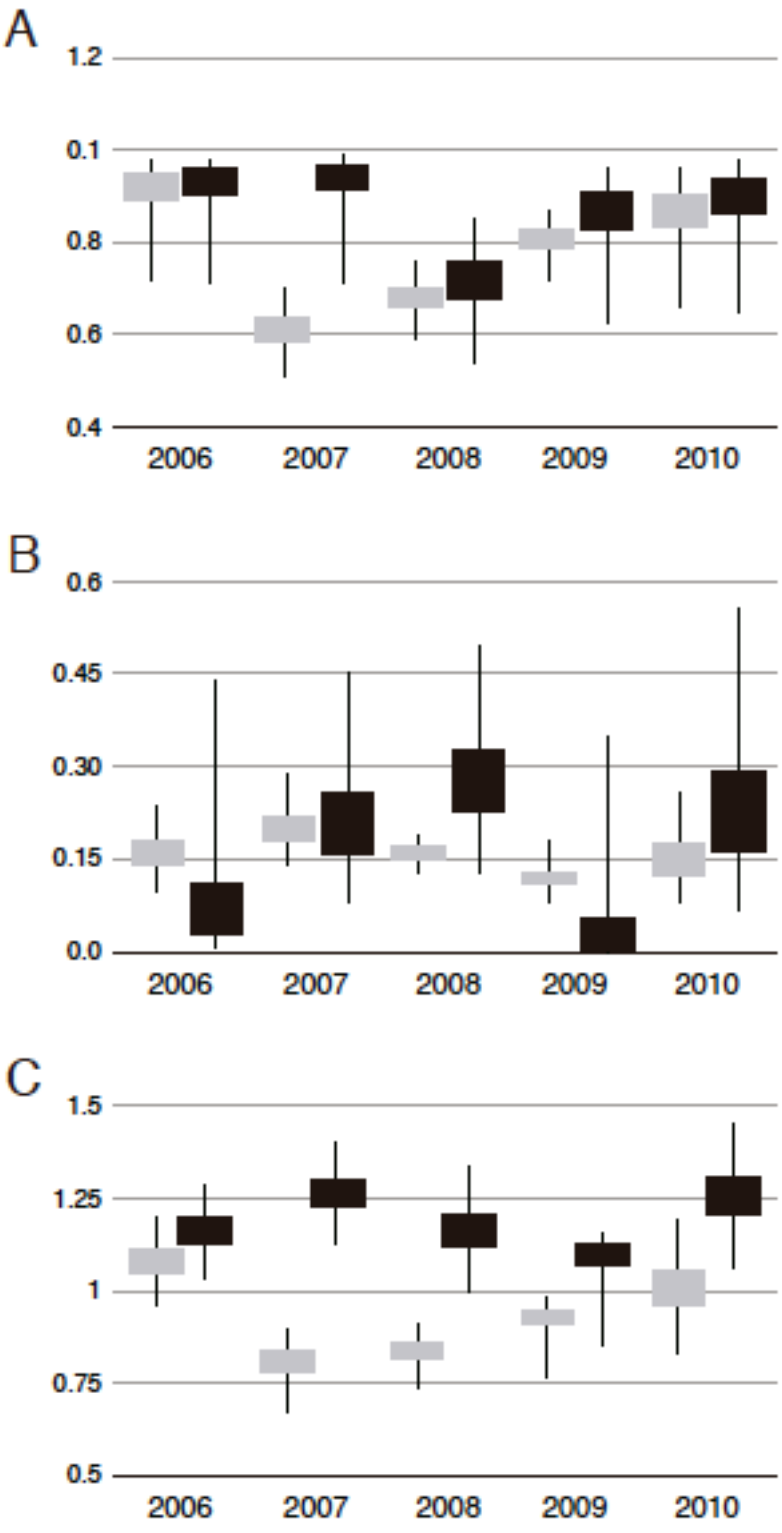

Figure 2. Model averaged estimates of probability of survival (A), recruitment (B) and population growth rate (C) for Laguna Grande (grey) and Laguna Chica (black). Estimates include standard error (SE) and 95% upper and lower confidence intervals.

SURVIVAL

The probability of adult survival at both Laguna Chica and Laguna Grande was relatively high (>0.75) although survival probability at Laguna Chica was generally higher (Fig. 2). While some disparity may be explained by variation in environmental conditions at the two ponds, common toads at GNP exhibited more variation in the probability of survival than expected relative to similar species (Pilliod *et al.* 2010, Muths *et al.* 2011). Variation in the probability of survival might also be a reflection of challenges in acquiring adequate food and shelter (i.e., life at the edge of a generalist's range). For example, common toads have a wide distribution in the Iberian Peninsula but are more likely to be found in areas where the climate (i.e., precipitation and temperature) is more predictable (Romero & Real 1996); it is possible that the extreme conditions potentially experienced at higher elevations promote variability in survival in Common Toads.

RECRUITMENT

In general food concentrations are low in the high-elevation, oligotrophic water bodies in GNP (Toro & Granados 1999). We might expect greater productivity at Laguna Chica compared to Laguna Grande because it is shallower and likely achieves higher solar gain. However, because common toad tadpoles are inefficient foragers with low ingestion and filtering rates when concentrations of food are low (Viertel 1990), this potential increase in production at Laguna Chica may not make a difference.

The idea of inadequate resources coupled with inefficient foraging ability has some support in that recruitment probabilities at the two ponds are generally lower (Fig.2) than recruitment rates reported for other toads in a similar environment (0.25-0.41, boreal toads, Muths *et al.* 2011). Lower recruitment could also reflect a response to a stressor such as disease. Mass mortalities of metamorphic common toads have been recorded in GNP since 2001 (Bosch & Martínez-Solano 2006, Garner *et al.* 2009), although high numbers of healthy metamorphs are observed as well (JB pers. obs.). The survival probability of metamorphic common toads has not been quantified but clearly affects recruitment rate.

Data on disease were collected only occasionally and thus limit our ability to test hypotheses relative to the effect of disease on demographic

parameters. However, the impact of *Bd* on the co-occurring midwife toad is acute (Bosch *et al.* 2001) such that an acknowledgement of a possible role of disease in common toad demography is appropriate. While common toads are not as susceptible to *Bd* as other species such as the midwife toad (Bosch *et al.* 2001, Bosch & Rincón 2008), density dependent factors can affect the interplay of disease and host (e.g., Rachowicz and Briggs 2007, Briggs *et al.* 2010) such that the Laguna Grande population (N > 300) may have reached a size enabling the fungus to compromise adult survival and thus population growth. The possibility of a density-dependent response to disease at Laguna Grande is plausible and could be tested rigorously with additional data.

Population growth

A relatively high survival probability is typical in anurans that live in unpredictable environments (e.g., at high elevations), thus we expect adult survival to be a key component of population growth (lambda) (Sather & Bakke 2000, Biek *et al.* 2002, Vonesh & De la Cruz 2002, Spencer & Janzen 2010). However, population growth is also determined by recruitment of adults. Recruitment may compensate for poor survival, maintaining a positive population trajectory or slowing a decline (e.g., Muths *et al.* 2011), potentially facilitating persistence in common toad populations despite fluctuations in breeding success. We expected to see the probability of recruitment at a level to maintain or increase the rate of population growth at Laguna Grande and Laguna Chica; instead we estimated a probability considerably lower than rates in other populations exposed to similar situations (extreme weather and disease) (Scherer *et al.* 2008, Muths *et al.* 2011).

Conclusion

There are many potential drivers of the scenario we describe at GNP. Consequences of disease dynamics and physiological consequences of living at the altitudinal extreme of the common toad's range provide two likely avenues of investigation. Changes in climate are expected to impact both weather patterns and potentially host-pathogen disease dynamics, but a longer time series of data may be necessary to fully investigate this.

It is prudent to remember that our data are only a snapshot in time and long-term dynamics of common toad populations at GNP may be very different. We demonstrate that short time series of data can be useful,

especially given the current state of amphibian affairs (e.g. Stuart *et al.* 2004), if assessed with the appropriate caveats.

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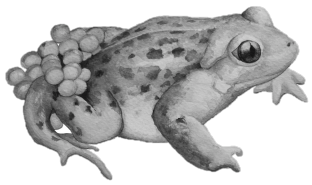

CAPÍTULO 9

SEGUIMIENTO A LARGO PLAZO DE UNA COMUNIDAD DE ANFIBIOS TRAS LA DESAPARICIÓN DE UNA DE LAS ESPECIES POR LA INTERACCIÓN ENTRE UNA ENFERMEDAD INFECCIOSA Y EL CAMBIO CLIMÁTICO

LONG-TERM MONITORING OF AN AMPHIBIAN COMMUNITY AFTER A CLIMATE CHANGE- AND INFECTIOUS DISEASE-DRIVEN SPECIES EXTIRPATION

SEGUIMIENTO A LARGO PLAZO DE UNA COMUNIDAD DE ANFIBIOS TRAS LA DESAPARICIÓN DE UNA DE LAS ESPECIES POR LA INTERACCIÓN ENTRE UNA ENFERMEDAD INFECCIOSA Y EL CAMBIO CLIMÁTICO

RESUMEN

Batrachochytrium dendrobatidis es un hongo patógeno introducido que ha provocado declives de anfibios a nivel global, cuya incidencia e impacto parecen estar muy relacionados con el cambio climático y las variaciones de temperatura. Los estudios realizados para explicar estos declives versan sobre los casos de mortalidad más llamativos, se centran en el estudio de una sola especie o se realizan a una escala temporal corta. En este estudio se aborda el seguimiento a largo plazo durante 18 años de una comunidad de anfibios completa, afectada por la quitridiomicosis, que es la enfermedad que produce el hongo. Para determinar el impacto del cambio climático, examinamos la relación entre las fluctuaciones de temperatura ambiental y los cambios en las abundancias de las diferentes especies de anfibios. Por otro lado, y para determinar si *Bd* continúa siendo una amenaza en el área, analizamos la prevalencia de la infección en la especie hospedadora más ampliamente extendida en la actualidad. Los resultados muestran que dos décadas después de la emergencia del patógeno las tendencias poblacionales de algunas especies continúan aparentemente estables o van en aumento, mientras que las especies más sensibles se encuentran en declive. La quitridiomicosis continúa estando presente y produciendo mortalidad en algunas especies, pero en niveles de infección mucho más bajos que durante los primeros años de emergencia del patógeno. Aunque la enfermedad parece ser la causa más influyente en la tedencia de la mayoría de las especies, los cambios climáticos parecen contribuir de manera significativa. A la vista de que las variaciones en la temperatura ambiental, asi como los rasgos biológicos y las interacciones bióticas entre especies modulan enormente la incidencia de la enfermedad, la realización de estudios regionales combinados resulta fundamental para poder predecir de forma más acertada la incidencia de la quitridiomicosis en una determinada comunidad de anfibios.

LONG-TERM MONITORING OF AN AMPHIBIAN COMMUNITY AFTER A CLIMATE CHANGE- AND INFECTIOUS DISEASE-DRIVEN SPECIES EXTIRPATION

ABSTRACT

Batrachochytrium dendrobatidis is an introduced fungal pathogen that has caused global amphibian declines, whose incidence and impact seem to be closely related to climate change and temperature variations. Most of studies conducted to explain these declines describe the most striking cases of mortality, are focused in a single species or are performed on a short time scale. We address the long-term follow-up for 18 years of a complete amphibian community, affected by chytridiomycosis, disease caused by the fungus. To determine the impact of climate change, we examined the relationship between environmental temperature fluctuations and changes in abundances of different amphibian species. To ascertain if *Bd* is a continued threat, we estimated prevalence of infection in the one remaining widespread amphibian species that is reported to be killed by lethal chytridiomycosis. The results show that two decades after the emergence of the pathogen, population trends of some species remain apparently stable or increasing, while the most vulnerable species are in decline. Chytridiomycosis remains in the area causing mortality in some species of the community but at levels of infection much lower than the first years of the pathogen's emergence. Although disease seems to be the most influential cause of most species' trends, climate changes appear to contribute also significantly. Since variations in environmental temperature, as well as biological features and biotic interactions between species, greatly modulate the incidence of the disease, the development of combined regional studies is fundamental to be able to predict more accurately the incidence of chytridiomycosis in a particular amphibian community.

INTRODUCTION

The current mass extinction stands apart from previous events due to the role a single species is playing in the reduction of global biodiversity (Ceballos *et al.* 2015). Human activities that modify habitats and alter ecosystem composition through introductions are behind contemporary biodiversity loss: evidence for this comes from decades of investigations into the causes of the global decline of amphibian biodiversity (Stuart *et al.* 2004, 2008). Habitat destruction, pollution and climate change render swathes of environments unsuitable for native amphibians, while introduced species and pathogens cause mass mortality or catastrophically impair recruitment (Berger *et al.* 1998, Davidson *et al.* 2002, Finlay & Vredenburg 2007, McMenamin *et al.* 2008, Johnson *et al.* 2011, Fisher *et al.* 2012, Faruk *et al.* 2013, Price *et al.* 2014). More worrisome is that many amphibian population declines or species extinctions have occurred in protected or relatively undisturbed areas (Collins & Crump 2009), illustrating the point that protected areas have no barriers to threats like climate change and infectious diseases.

Despite the increasing abundance of reports of amphibian declines, it is likely that these are inaccurately informing us as to the impacts of climate change, infectious diseases or their combination. This is because publications generally report worst-case scenarios, where impacts are immediate, overt and measurable over short time scales (Berger *et al.* 1998, Bosch *et al.* 2001, Lips *et al.* 2006, 2008, Price *et al.* 2014, Stegen *et al.* 2017). While some of these studies report taxonomically broad impacts, others describe demographic declines of single species in multi-species amphibian communities, or spatially heterogeneous population responses (Bosch *et al.* 2001, Lips *et al.* 2006, 2008, Vredenburg *et al.* 2010, Walker *et al.* 2010, Puschendorf *et al.* 2013, Price *et al.* 2014, Price *et al.* 2016). These inconsistencies suggest that the threat posed by climate change and infectious diseases are not comprehensively described by short-term studies, which long-term monitoring projects are starting to reveal. For example, in some communities impacts are likely being underestimated, as cryptic, subtle, indirect or post-decline spillover effects that cause population declines downstream from the initial mortality event are now being reported (e.g. Scheele *et al.* 2016, Clare *et al.* 2016). Alternatively, some populations that have experienced climate- and/or disease-driven declines appear to be recovering, suggesting that in some cases threat may be overestimated (Scheele *et al.* 2015, Knapp *et al.* 2016).

Batrachochytrium dendrobatidis (hereafter *Bd*) is a generalist, chytridiomycete fungal pathogen responsible for amphibian declines and extinctions at a global scale (Berger *et al.* 1998; Fisher *et al.* 2009). Climate change, predominantly differences in environmental temperatures, has been invoked as an important cofactor influencing the degree of impact chytridiomycosis caused by *Bd* may have on amphibian populations and communities (e.g. Walker *et al.* 2010, Cohen *et al.* 2017). The assumption that temperature dictates the virulence of *Bd* epidemics is predicated on laboratory *in vitro* studies of temperature-dependent *Bd* growth rates. Macroecological and other studies investigating the 'chytrid thermal optimum hypothesis' report ambiguous or contradictory findings, or suggest a more direct effect of climate change (Pounds *et al.* 2006, Lips *et al.* 2008, Rohr *et al.* 2008, Rohr & Raffel 2010). Indeed, climate change is likely behind many amphibian species population declines and range contractions, and associated changes in parasite incidence and prevalence, although affected by changes in environmental metrics, may be coincidental and not causative. Nearly all of these studies invoke issues of scale and suggest the impact of climate, chytridiomycosis and their interactions will be elucidated through long-term amphibian population and community monitoring projects.

Europe is home to several such long-term amphibian monitoring projects, including the SOS Anfibios Guadarrama project based in the Peñalara Massif at the Sierra de Guadarrama National Park. SOS Anfibios was the first project to detect European amphibian declines due to *Batrachochytrium dendrobatidis*, in this case affecting the common midwife, *Alytes obstetricans* (http://www.parquenacionalsierraguadarrama.es/es/blogs/sos-anfibios, Bosch *et al.* 2001) and subsequently described for this species in 3 other European countries (Walker *et al.* 2010, Tobler & Schmidt 2010, Rosa *et al.* 2013). The disease-driven near-extirpation of common midwifes on the Peñalara Massif has since been attributed to a threshold effect of increasing environmental temperature on the emergence of chytridiomycosis (Bosch *et al.* 2007). Two other amphibian species have been reported dying from chytridiomycosis caused by *Bd* and potentially declining as a result (Bosch & Martínez-Solano 2006, Garner *et al.* 2009). However, population and *Bd* transmission dynamics of these two other species years after the initial disease emergence event suggest that early population responses do not fully describe demographic response to changing climate and disease in the region (Bosch & Rinçon 2008, Medina *et al.* 2015, Fernández-Beaskoetxea *et al.* 2016).

Here, we report the results of long-term monitoring of the amphibian community in Peñalara 18 years after the disease outbreak. We examine the relationship between fluctuating environmental temperature and changes in amphibian species abundance to determine the impact climate change is having on host community structure. In conjunction with this and to ascertain if *Bd* is a continued threat, we estimated prevalence of infection in the one remaining widespread amphibian species that is reasonably common and reported to be killed by lethal chytridiomycosis, the fire salamander *Salamandra salamandra* (Bosch & Martínez-Solano 2006, Medina *et al.* 2015), and examined if prevalence of infection had downstream effects on the abundance of larval salamanders.

METHODS AND MATERIALS

The Peñalara Massif (Guadarrama Mountains National Park, Central Spain, near Madrid, 41°N, 4°W) is an alpine (1800-2430 m) habitat with 242 ponds above the tree line (Toro & Granados 1999). Ponds lack vegetation along the shoreline, range in area from 0.3 to 6463.4 m^2 and are clustered (1-24 ponds per cluster) within 29 basins (hereafter, sectors). The landscape consists mainly of granitic outcrops, alpine meadows, heathlands dominated by *Cytissus oromediterraneus* and *Juniperus communis nana*, and forests of *Pinus sylvestris* below the timberline. Nine amphibian species breed regularly in the area, and the combination of lithology and scarcity of aquatic vegetation provides crystal-clear waters that are excellent for visual surveys of larval abundance (Martínez-Solano *et al.* 2003).

We comprehensively surveyed all ponds across the Peñalara Massif during the amphibian reproductive and development period (May to September) each year from 1999 and 2016. For each species, we recorded number of clutches (for bufonids) or estimated larval abundances (all other species) up to 6 times per season and per pond, dependent on the hydroperiod and associated persistence of ponds. In larger ponds, or at high tadpole densities (roughly >100), we estimated the number of tadpoles through sub-sampling (stratified when required by habitat diversity) by counting all the larvae present in predetermined areas and extrapolating to the entire pond. Only maximum counts per year were considered. These maximum counts represent the absolute number of larvae or clutches per pond after the peak of the reproductive period for each species was reached, which was ensured by repeated visits throughout the breeding season. To standardize minor variations in counting, and to minimize sampling error, we used six classes with different break points to score

larval abundance (0, 1–10, 11–50, 51–100, 101–500, and >500) and number of clutches (0, 1, 2-5, 6-10, 11-20, >20). The median counts of the different ponds of each sector were added for each year to obtain a single annual estimate. Thus, the data for each species were summarized in 29 independent cumulative counts per year. These data of 29 sectors during 18 years per species were used in the TRIM analyses (see below). The large number of ponds in which the 29 study sectors were divided favoured that only a very small proportion of the counts were included in the broader and larger count class throughout the 18 study years (242 ponds x 18 years = 4356 different counts): *Alytes obstetricans*, 0.02%; *Bufo calamita*, 1.03%; *Bufo spinosus*, 0.23%; *Hyla molleri*, 0.67%; *Ichthyosaura alpestris*, 0.34%; *Pelophylax perezi*, 0.39%; *Rana iberica*, 0.00%; *Salamandra salamandra*, 0.53%; *Triturus marmoratus*, 0.00%.

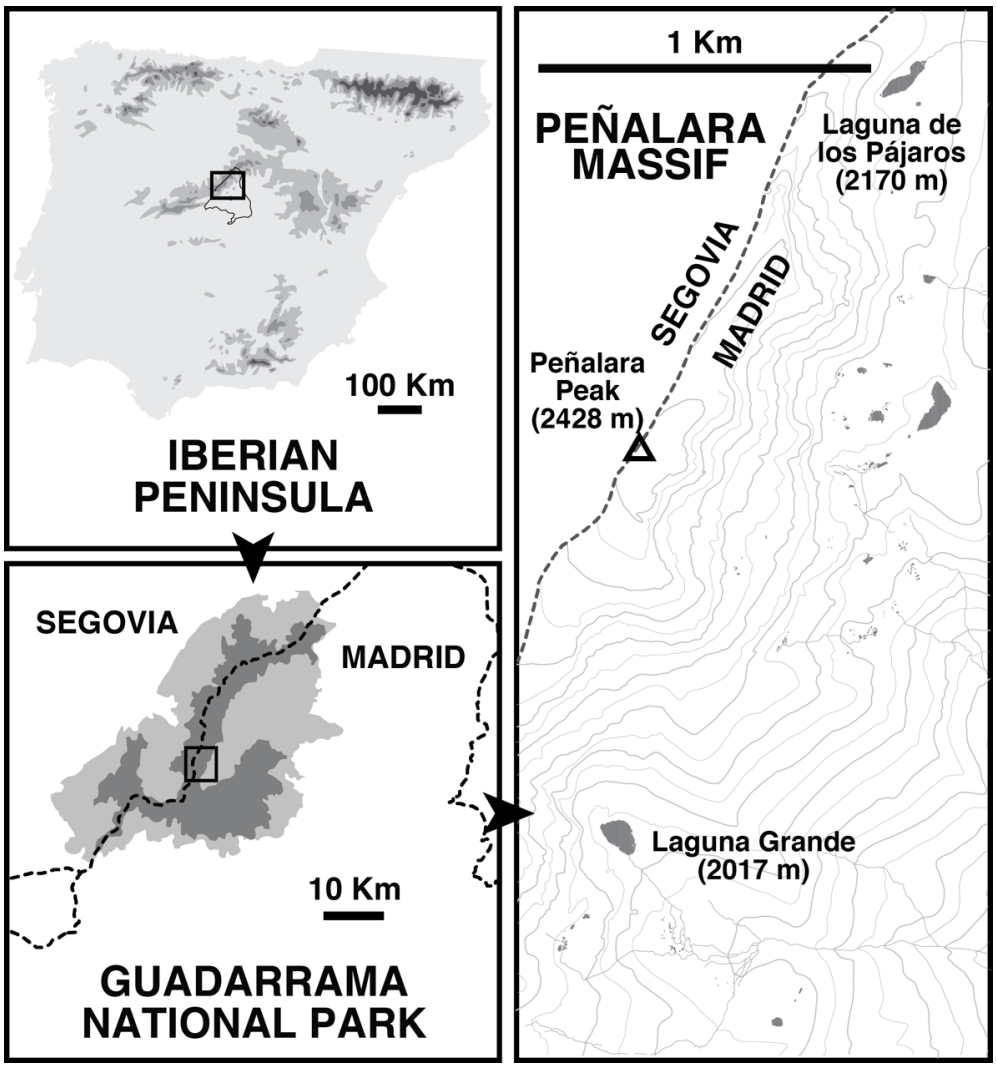

Figure1. Location of the studied breeding ponds within Guadarrama National Park in the Iberian Peninsula.

To assess the relationship between temperature variation and amphibian abundance we used ambient minimum and maximum temperature data generated at the nearby meteorological station in Cotos mountain pass (provided by Guadarrama National Park, 1857 m a.s.l). We used data sets from 1991 to 2016 in order to estimate how time spans of up to eight years preceding affected the annual abundance of each species. As monthly minimum and maximum temperature averages were highly and positively correlated throughout the whole study period (R^2 = 0.972, n= 312 months in 26 years), we focussed on two more distinct temperature measurements for analyses: yearly minimum temperature of the coldest month (January or February depending on the years) and yearly maximum temperature of the hottest month (July or August depending on the years; Figure 2). These two variables represent the most extreme thermal conditions within the year but were weakly and negatively correlated across the 26 years of temperature records (R^2 = 0.270, n = 26). The yearly minimum and maximum temperatures were separately averaged for eight time spans of different length, from one (the census year) to eight consecutive years (from the year of the census to seven years before). The correlation between the minimum and maximum temperatures were very low and non-significant in the eight time spans (from a minimum R^2 of 0.018 for the time span of one year, to the maximum R^2 of 0.084 for the time span of eight years). We saw no evidence of temporal autocorrelation in the linear trend autocorrelation models of yearly minimum temperature and yearly maximum temperature of the census year (as shown by the autocorrelation function plots), suggesting that the time series was stationary around a deterministic trend and that differencing is not required (i.e., the statistical properties of the time series of the minimum and maximum yearly temperatures in 1999-2016 were constant over time).

DATA ANALYSES

Annual population trends were estimated using the time-effects model of TRIM, statistical software developed specifically for time series of animal counts in monitoring programmes (Pannekoek & van Strien, 2005). Specifically, we developed log-linear Poisson regression models from species' counts using years (1999–2016) as the continuous predictor. These models accounted for over-dispersion and serial autocorrelation in the data to obtain population trends (±1) as the slope of the regression of the logarithms of the yearly indices. Standard errors of the trends were estimated as a measure of uncertainty in average linear population trends. We did not use non-linear models as our objective was to investigate overall

change during the course of the sampling period and not, annual or other short-term fluctuations in abundance (see also Heldbjerg & Fox 2008, Gregory *et al.* 2009, and Reif *et al.* 2011 for a similar approach). Linear trend TRIM models were compared with their corresponding null TRIM models (not including the linear effect of year) using Akaike's Information Criterion (AIC). We estimated the overall additive change for each species by measuring the average inter-annual rate from the first to the last year of the study (Table1).

We constructed AutoRegressive Integrated Moving Average (ARIMA) models to estimate the influence of annual maximum and minimum temperatures on annual amphibian species abundance. Sixteen ARIMA models were built for each species using the command *auto.arima* of the package forecast run under R version 3.1.2 (R Core Team 2014): eight each using the yearly maximum or yearly minimum temperatures for one and up to cumulative years preceding the sampling year. We used the *auto.arima* command and the logarithm of the amphibian counts to obtain the optimum p, d and q parameters in ARIMA models. Second order Akaike's AICc for finite sample sizes were used to obtain the weights of these sixteen models. Model weights within species were added according to two variable categories: (1) minimum vs. maximum temperatures (sum of eight weights in each category), and (2) eight time spans (sum of two weights -maximum and minimum temperatures-per time span from the census year to seven years before the amphibian counts). To illustrate the strength and significance of temperatures on species-level count data, we listed the ARIMA model with the lowest AICc value (Table 2). We tested for temporal autocorrelation using the residuals of the selected ARIMA models and autocorrelation function plots, the Kwiatkowski–Phillips–Schmidt–Shin (KPSS) test and the Ljung–Box test (up to lag six years), with the commands *Acf* of the package forecast (Hyndman 2016) and *kpss.test* of the package tseries (Trapletti *et al.* 2017), and *Box.test*. No trace of temporal autocorrelation was found in the residuals of the ARIMA temperature-models of the studied nine species. We used principal component analysis (PCA) of the logarithm of the abundance data for each species and each year to illustrate any general patterns of population trends across species. We employed a 5-fold cross-validation method for determining the optimal number of principal components, using Statistica 12 (StatSoft. Inc.).

Although all amphibian species inhabiting the Peñalara Massif can become infected with *Bd*, only 3 species have been reported to experience mortality due to chytridiomycosis (Bosch *et al.* 2001, Bosch & Martínez-

Solano 2006, Garner *et al.* 2009). Of these, *Alytes obstetricans* is for all intents and purposes extirpated in the area, while the distribution of *Bufo spinosus* is restricted to a few, permanent ponds located in different drainages of the massif. Because we were interested in the impacts of spatiotemporally broad impacts of *Bd* on resident species, we focussed on the potential impacts of infection on the remaining species that occupies a broad distribution in the massif and was most likely to exhibit changes in abundance associated with chytridiomycosis, *Salamandra salamandra.* To do this we used generalized least squares regression models (GLS), taking into account any potential temporal autocorrelation of data. We calculated prevalence of infection of larval and metamorphosed salamanders per year and modelled the impacts of these estimates on larval abundance from 1 to a maximum of 7 years after sampling for infection was completed. We began sampling (swabs or tissue samples) *S. salamandra* starting in 2001 and used larval counts from 2001 to 2016. The average number of samples per year was 85.5 and comprised the whole study area but was patchly distributed across years and ponds. We assigned infection status used qPCR following Boyle *et al.* 2004. As a result the number of comparisons per time span ranged from a maximum of 16 for the one-year period to 9 for the eight-year time span. Predictors for GLS models were the temperature measurement from the best-fit ARIMA models listed in Table 2 and prevalence of adult salamanders in the time spans that most strongly correlated with larvae counts. GLS models were carried out using the gls command of the nlme package and temporal autocorrelation structure of data was taken into account using the corARMA argument (Pinheiro *et al.* 2017).

RESULTS

The counts for all nine amphibian species have shown marked fluctuations from 1999 to 2016 in Peñalara (see Figure 2 for changepoints in inter-annual differences between consecutive years according to a stepwise selection approach), and the goodness of fit likelihood ratio tests show that the null models (i.e., stable population patterns) do not fit the data ($p<<0.001$ in all cases). Serial correlations (Table 1) attain low figures for *I. alpestris*, *S. salamandra* and *T. marmoratus* ($r < 0.3$), but medium values for the remaining species (r: 0.35-0.53), denoting that the counts in a particular year depended to some amount on the counts in the year before. Population trend models assuming a monotonic increasing or decreasing trend throughout the 18 years of study (LINEAR models) have a higher strength of evidence than the NULL models in all species, except in *B.*

calamita and *P. perezi*. *Alytes obstetricans*, *R. iberica* and *S. salamandra* showed highly significant negative linear trends of counts with time, while *H. molleri*, *I. alpestris* and *T. marmoratus* manifested a highly significant increase with time (see slopes in Table 1).

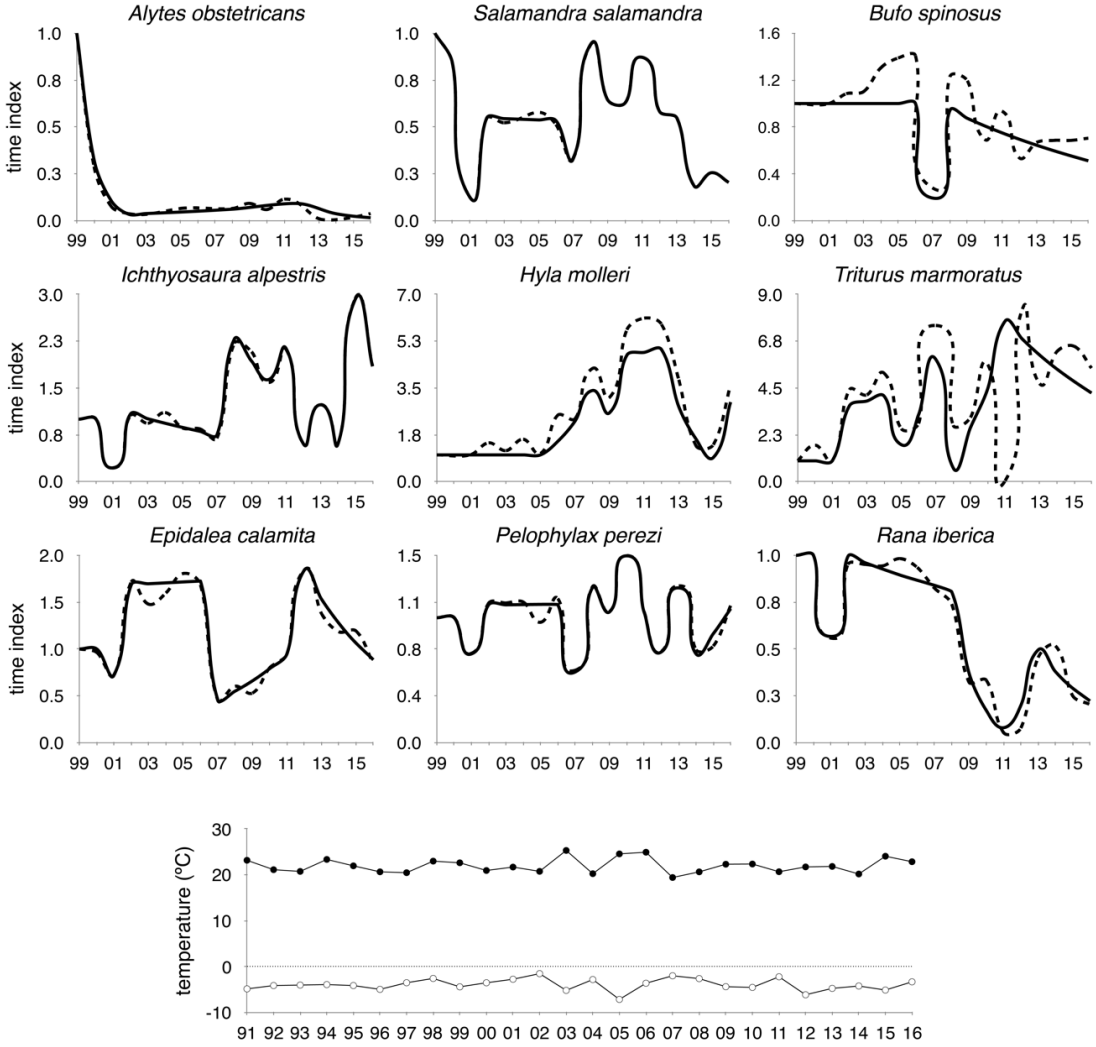

Figure 2. Population indices of nine amphibian species in Peñalara from 1999 to 2016 in 18 consecutive years. Time index 1 refers to the amphibian counts measured in 1999 (i.e., the baseline in first sampling year). Continuous lines show the measured trends in the amphibian counts per year, while the discontinuous ones are established by means of TRIM models (TRend analysis and Indices for Monitoring data) using stepwise selection of changepoints where changes in slope between consecutive periods of time attained significance. Recall that there are periods when both types of lines overlap. Monthly average maximum temperatures of the hottest month (usually July or August of each year; black circles), and monthly average minimum temperatures of the coldest month (usually January or February of each year; open circles), from 1991 to 2016 are presented in the lower part of the figure.

For the whole study period, *H. molleri*, *I. alpestris* and *T. marmoratus* experienced positive and significant average interannual population trends, with yearly increases ranging from 5.1% to 9.2% (Table 1; see also Figure 2, paying attention to the fact that the fluctuations of the relative time index is above 1 – i.e., the population level recorded at the beginning of the study period). Conversely, *R. iberica* and *S. salamandra* suffered significant average declines of -11.2% and -3.5% respectively (see Figure 2). Although *A. obstetricans* experienced a striking population decrease since 1999, its average interannual additive parameter is not significantly different from cero in spite of its high negative value of -12.9% (p = 0.095). This is easily explained considering that the population of *A. obstetricans* in Peñalara collapsed from 1999 to 2002 (with a deep 97% decrease), then its population level increased 162% from 2002 to 2012, and finally the population trend was again negative. The remaining species did not show clear population trends throughout the study period, although *B. spinosus* population has slightly decreased, especially since 2008. Steep decreases or increases in population indices do not show generalizable consistencies across species.

Table 1. Results of TRIM models analyzing count data obtained from monitoring amphibian populations of nine species in Peñalara during 18 consecutive years (May-August 1999-2016) in 29 different sampling sectors (for details see the Methods section).

		AICc		Linear model			Additive change		
	Serial r	Null	Linear	Slope	se	p	Parameter	se	p
A. obstetricans	0.422	6648	4294	-0.274	0.074	<0.001	-0.129	0.007	0.095
B. calamita	0.430	2876	2873	-0.006	0.010	0.530	-0.005	0.009	0.594
B. spinosus	0.400	225	202	-0.030	0.016	0.066	-0.034	0.019	0.065
H. molleri	0.532	46686	42758	0.069	0.022	0.002	0.077	0.017	<0.001
I. alpestris	0.188	22602	20323	0.051	0.016	0.001	0.051	0.018	0.005
P. perezi	0.350	23050	23053	0.000	0.012	0.988	-0.002	0.014	0.877
R. iberica	0.363	9019	7461	-0.086	0.029	0.003	-0.112	0.032	0.001
S. salamandra	0.270	59588	57128	-0.039	0.009	<0.001	-0.035	0.011	0.001
T. marmoratus	0.185	2583	2354	0.063	0.023	0.006	0.092	0.030	0.003

Two different models have been built for each species: no time effect models (NULL; testing for the absence of population trends) and LINEAR trend (assuming an increasing or decreasing monotonic trend). Serial r: serial correlation measuring the association of the counts in year *t* with year *t*-1. The AIC figures are provided for each one of these models. It is provided the slope of the LINEAR trend model, with the standard error and significance (against Ho: slope=0). The ADDITIVE parameter defines the overall additive change measured in the data for each species (with its standard error –se– and the

significance of deviation from the null hypothesis of 0 –p–). Population counts remain constant throughout time if the additive parameter equals zero; an additive figure of -0.112 denotes that from the first to the last year of amphibian counts the population has decreased at an average inter-annual rate of 11.2% (-0.112*100). All significant tests remain significant after Bonferroni sequential correction.

ARIMA models built with different temperatures and time lags previous to the census year have different influences on the nine studied species (Table 2). Minimum temperatures were very influential in *B. spinosus*, *R. iberica* and *T. marmoratus*, while maximum temperature was very influential in *E. calamita*; for the remaining species no clear trend was observable (highest weight sums lower than 0.7). On average, the most influential time span in which temperatures were averaged was six years (from the census year to five years before). ARIMA models with significant temperature effects were obtained for six species (after sequential Bonferroni adjustment to control for type I error in the nine significance estimations). The partial effects attributable to temperature (partial R^2), after controlling for the serial autocorrelation, were relatively low for all species (in brackets after the name of the species). The larvae count for *H. molleri* (partial R^2 = 0.150 with minimum temperature), *I. alpestris* (0.040, maximum temperature), *S. salamandra* (0.133, maximum temperature) and *T. marmoratus* (0.165, minimum temperature) significantly increased with average year month temperature during the first six or eight years. Conversely, the number of clutches of *E. calamita* (partial R^2 = 0.096) and larvae of *R. iberica* (0.134) decreased with increasing, respectively, the maximum and minimum temperatures during the six years before the counts.

Table 2. Results of ARIMA models testing for the influence of temperature on yearly trends of amphibian counts in Peñalara.

	Min	Max	One	Two	Three	Four	Five	Six	Seven	Eight	Temp	Sign	p
A. obstetricans	0.34	0.66	0.12	0.06	0.06	0.06	0.09	0.29	0.22	0.10	max-6	+	0.060
B. calamita	0.22	0.78	0.11	0.05	0.06	0.09	0.19	0.35	0.10	0.06	max-6	-	0.003
B. spinosus	0.71	0.29	0.09	0.13	0.11	0.20	0.11	0.10	0.12	0.15	min-4	+	0.044
H. molleri	0.54	0.46	0.05	0.20	0.06	0.04	0.05	0.27	0.23	0.10	min-6	+	0.005
I. alpestris	0.41	0.59	0.09	0.08	0.11	0.10	0.12	0.25	0.13	0.12	max-6	+	0.000
P. perezi	0.57	0.43	0.08	0.29	0.14	0.13	0.09	0.09	0.09	0.09	min-2	+	0.070
R. iberica	0.81	0.19	0.05	0.03	0.03	0.05	0.73	0.05	0.03	0.03	min-5	-	0.001
S. salamandra	0.42	0.58	0.04	0.12	0.06	0.08	0.10	0.14	0.17	0.29	max-8	+	0.000
T. marmoratus	0.80	0.20	0.14	0.11	0.03	0.10	0.09	0.37	0.05	0.10	min-6	+	0.003

Temperature measurements include the average minimum temperatures of the coldest month (usually January or February depending on the year) and average maximum temperatures of the hottest month (usually on July or August), with time lags of one to eight years before the year when the amphibians were censused (i.e., 1, 2, 3, 4, 5, 6, 7 and 8 years before). The figures for minimum and maximum temperatures are the addition of the AICc weights of the eight ARIMA models built, respectively, with the minimum and maximum yearly temperatures. The figures for one, two, ... eight years before the amphibian counts refer to the addition of the AICc weights of the two ARIMA built with the minimum and maximum temperatures for each time span. TEMP: the model of highest evidence including a measure of temperature (i.e, lowest AICc and highest weight values). P and sign: significance and sign of the regression coefficient of temperature on the amphibian counts considering the corresponding time span. min-2, min-4, min-5 and min-6: average minimum temperature of the coldest month during the previous two, four, five and six winters before the year of the amphibian counts, respectively. max-6 and max-8: average maximum temperature of the hottest month during the previous six or eight years of the amphibian counts, respectively.

A principal components analysis with the amphibian annual counts of the nine species during the 18 study years produced one significant component obtained by simple cross-validation. This component accounts for 31.8% of the information content of the correlation matrix among the nine species (eigenvalue = 2.861). The species with the highest absolute factor loadings in this component are *R. iberica* (0.718), *H. molleri* (-0.896), *T. marmoratus* (-0.854) and *I. alpestris* (-0.676; the threshold for factor loadings was stablished arbitrarily at +/-0.590, the correlation coefficient at p = 0.01 and n = 18). These four species are responsible of 87% of the informative content of this component. Summarizing, *H. molleri*, *T. marmoratus* and *I. alpestris* shared a relatively similar pattern of covariation of population increase from 1999 to 2016, while *R. iberica* showed an inverse pattern of population decline.

Discussion

While global assessments on host trajectories after chytridiomycosis outbreaks at regional or continental scales are essential to identify patterns and mechanisms of species responses to *Bd*, precise analyses at the local scale are also very valuable. Actually, besides chytridiomycosis direct effects over every single species, interspecific differences in its impact may alter assemblage-influencing biotic interactions and, therefore, assemblage structures in indirect ways that can not been assessed by regional anaylses

(Bosch & Rincón 2008). Moreover, detailed studies of populations, as ours, could be essential to develop effective measures to reduce chytridiomycosis impacts because individual patterns and mechanisms of species responses can be easily inferred. Our study place, with nine species sharing about 250 breeding ponds into a small area is an excellent system to investigate processes that shape long-term, variable host responses to a generalist pathogen as is *Bd*. Our general results match similar ones at broader scales (as Scheele *et al.* 2017) founding highly variable long-term impacts on species trajectories. That is, two decades post-emergence, some species are apparently stable or increasing, while other species are in an ongoing state of decline, and the same species remain in the area from the 80's (Martínez-Solano *et al.* 2003). Even when there are many potential drivers of the general scenario we describe here, disease dynamics seems to be the most plausible for the majority of the species, although changes in climate have a significant contribution explaining some trends.

While in tropical areas chytridiomycosis-induced adult mortality is usually high in very susceptible species, recruitment can compensate loss, resulting in persistent, but seasonally fluctuating populations (e.g. Murray *et al.* 2009; Phillott *et al.* 2013; Scheele *et al.* 2015). In Peñalara, mass mortalities associated with *Bd* are almost restricted to recent metamorphosed animals, resulting in delayed, but inexorable, declines. *Alytes obstetricans*, the most susceptible species to *Bd*, has not been completely extirpated after 6 consecutive years of dramatic population crashes (1997-2001). However, despite reintroduction efforts (above 500 metamorphosed animals released on four locations across the park) carried out from 2008 to 2013 (Bosch *et al.* unpublished data), just a small new population was stablished around a trough, and the species remains in both low and high elevations of the park during the last 15 years but in steamily low numbers. The second most susceptible species, *S. salamandra*, started suffering mass mortalities four years after initial chytridiomycosis emergence (Bosch & Martínez-Solano 2003) and still abundant despite its remarkable decline during the whole studied period of 18 years. The plasticity exhibited for the species, occupying also temporary water bodies and streams where *Bd* survival is low or transmission probability is poor, is probably preventing a more dramatic situation. Finally, the third species more susceptible to *Bd*, *B. spinosus*, not show a clear population trend throughout the studied period, but its population slightly decreased since 2008. Actually, a more powerful population analyses performed by mark-recapture from 2006 to 2010 found a negative population growth rate at the central breeding pond due to an inadequate recruitment to compensate

for the survival rate (Bosch *et al.* 2014). This, together with its initial expansion to 5 ponds vacated by the extirpation of *A. obsteticans* (Bosch & Rincón 2008) explains its divergent trend over the whole studied period. Although some dead recent metamorphosed *B. spinosus* specimens were observed from 2001, they become massive from 2005, seven years after initial chytridiomycosis emergence, and still abundant at the present when *S. salamandra* mortalities are now scarce. Therefore, the three more susceptible species experienced successive population crashes and declines, even though biotic interactions and biological features are modulating their relevance.

B. calamita and *P. perezi* remain stable throughout the studied period. Both species are known to be good host for *Bd* (Baláz *et al.* 2013), but habitat-related indirect effects as well behavioural features are probably preventing extreme consequences. *B. calamita* breeds on temporary or ephemeral ponds where water temperatures at the end of the metamorphosis prevent high infections, and *P. perezi* exhibits basking habits that can reduce infection levels. In fact, our results show that maximum temperature was very negative for *B. calamita* since clutches and tadpoles can die by increased evaporation and the lengthened period of drought in such very shallow ponds use by the species.

On the other hand, three species increased their numbers in Peñalara after the chytridiomycosis outbreak. The expansion of *I. alpestris* before (Martínez-Solano *et al.* 2003) and throughout the studied period can be easily explained by its allochtonous character, wich have been recently demonstrated (Palomar *et al.* 2017). *Hyla molleri* and *T. marmoratus* inhabit low altitude areas in the Iberian Peninsula, and both started colonizing the area in the late 80's (Martínez-Solano *et al.* 2003) matching the sharp temperature increase registered then. Additionally, population changes of these three species can be related to thermal fluctuations throughout the studied period, indicating their dependency with temperature under alpine conditions existing in the studied area. Expanding species shared a relatively similar pattern of covariation of population increase despite their presumably distinguish outcomes, while *R. iberica* showed an inverse pattern of population decline.

Actually, the sharp decline recorded for *R. iberica* is intriguing and probably not related to *Bd*. Despite larvae can be experimentally infected with *Bd* (Fernández-Beaskoetxea *et al.* 2016) and overwintering larvae tested positive in natural conditions (Bosch *et al.*, unpublished data), dead

animals were never recorder. It must be remarked that main breeding habitats for this species have not tacked into account in this study. *Rana iberica* reproduces mainly on streams, so larval counts in ponds are not reflecting its real population size but representing residual populations persisting in suboptimal breeding sites inaccessible to fish (Bosch *et al.* 2006). Therefore, since the species shows an inverse pattern of covariation with expanding species, it is possible that interspecific competition is explaining its negative trend found in ponds. Additionally, counts of *R. iberica* decreased with increasing minimum temperatures of previous years. Therefore, probably warmer winters throughout the studied period could have contributed to deteriorate the conditions on such suboptimal breeding sites for this montane species.

Infection levels in Peñalara have been decreasing throughout the studied period together with the near extirpation of *A. obstetricans*. In 2005, infection was remarkable in more than 60% of ponds (Walker *et al.* 2007), while in 2016 infection was practically absent on temporal ponds and just remains present in a few permanent ponds (Serrano-Laguna *et al.* unpublished data). At the present, *A. obstericans* remains only in interconnected ponds and small rivulets where water flow reduces the density of zoospores available for transmission reducing the likelihood of successful transmission. *Salamandra salamandra* overwintering larvae at permanent ponds are now driving infection dynamics in this system (Medina *et al.* 2015), maintaining infection from year to year and transmitting infection to young of the year salamander and *B. spinosus* larvae.

Appendix. Counts of clutches (for *Bufo spinosus* and *E. calamita*) or larvae (the remaining species) in 29 different high altitude sectors in Peñalara (Guadarrama National park). *Alytes obstetricans* (Ao), *Epidalea calamita* (Bc), *Bufo spinosus* (Bs), *Hyla molleri* (Hm), *Ichthyosaura alpestris* (Ia), *Pelophylax perezi* (Pp), *Rana iberica* (Ri), *Salamandra salamandra* (Ss), *Triturus marmoratus* (Tm).

Year	Ao	Ec	Bs	Hm	Ia	Pp	Ri	Ss	Tm
1999	1144	283	54	750	1490	2628	795	9199	24
2000	330	272	54	756	1496	2634	795	7788	42
2001	86	204	54	762	326	1878	444	1179	24
2002	43	485	59	1087	1578	2934	756	5023	106
2003	43	418	60	869	1393	2940	756	4805	100
2004	49	452	71	1188	1648	2959	750	5068	125
2005	74	510	75	789	1282	2534	781	5349	61
2006	74	476	76	1862	1261	3031	744	4723	67

Year	Ao	Ec	Bs	Hm	Ia	Pp	Ri	Ss	Tm
2007	86	128	15	1762	1035	1522	656	3023	180
2008	74	170	67	3169	3299	3277	588	8619	305
2009	105	150	65	2363	3099	2761	257	5919	74
2010	68	226	38	4257	2364	3935	265	5773	135
2011	131	268	51	4581	3156	2754	43	8016	241
2012	92	520	29	4408	901	1923	62	5356	199
2013	12	390	36	2856	1838	3301	361	5037	113
2014	12	335	37	1004	901	1957	411	1701	150
2015	18	337	37	1035	4366	2033	199	2353	156
2016	43	237	38	2725	2762	2886	162	1875	131

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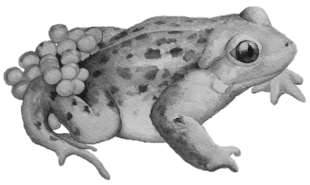


CONCLUSIONES/CONCLUSIONS

CONCLUSIONES

1. Las actividades humanas resultan clave en la diseminación del hongo patógeno de los anfibios y, dado que las vías existentes para su dispersión son múltiples a todos las escalas espaciales, es esperable que la quitridiomicosis acabe siendo enzoótica de cualquier lugar donde se den las condiciones ecológicas mínimas para la supervivencia del patógeno.

2. En zonas templadas, la temperatura es el factor más importante para explicar la incidencia de la quitridiomicosis. Por lo tanto, la intensidad y la prevalencia de infección presentan una clara variación estacional, siendo más elevadas durante los meses más fríos del año.

3. El estadio de desarrollo durante el cual se infectan los anfibios resulta clave para el desarrollo y las consecuencias de la quitridiomicosis. Por otro lado, la infección de los ejemplares puede mantenerse durante largos períodos de tiempo sin que sea necesario ninguna fuente externa de reinfección.

4. Como cabría esperar, las mortalidades masivas producidas por la quitridiomicosis comprometen seriamente la variabilidad y estructura genética de las poblaciones. Por lo tanto, y con objeto de mantener la mayor diversidad genética original posible, los programas de reintroducción deben apoyarse en estudios genéticos que permitan cruzamientos óptimos.

5. La diferente susceptibilidad de las especies de anfibios a la quitridiomicosis, hace que, tanto la dinámica de transmisión, como la intensidad de la infección, varíen en función de la composición de especies de la comunidad, resultando muy difícil predecir el efecto que produciría la llegada del patógeno a un determinado enclave.

6. Algunas especies de anfibios se comportan como super-hospedadoras, aumentando de forma desproporcionada la infección de otras especies presentes en la comunidad.

7. Los programas de marcaje y recaptura de anfibios, aunque desarrollados durante períodos relativamente cortos, permiten obtener con precisión parámetros demográficos tales como la tasa de supervivencia o la tasa de reclutamiento, y establecer tendencias

poblacionales. Sin embargo, esta información debe ser contextualizada con factores abióticos y bióticos, dado que las condiciones meteorológicas y la quitridiomicosis pueden influir también en las dinámicas poblacionales.

8. Las especies sensibles a la quitridiomicosis experimentan declives poblacionales muy acusados, y prolongados en el tiempo, que varían en intensidad según su diferente grado de susceptibilidad a la enfermedad. Sin embargo, y al menos en zonas altas de montaña, el cambio climático determina frecuentemente las tendencias poblacionales, siendo necesario un análisis combinado para establecer de forma precisa la incidencia de la enfermedad.

CONCLUSIONS

1. Human activities play a crucial role in the dissemination of the pathogenic fungus of amphibians and, given the multiple pathways that exist for their dispersion at all spatial scales, it is expected that chytridiomycosis becomes enzootic in every places with the minimal ecological conditions for the survival of the pathogen.

2. In temperate zones, temperature is the most important factor to explain the incidence of chytridiomycosis. Therefore, the intensity and prevalence of infection show a clear seasonal variation, being higher during the coldest months of the year.

3. Developmental stage in which amphibians are infected is key to the incidence and consequences of the outcome of the disease. On the other hand, infection of the specimens can be maintained for long periods of time without the need of any external source of reinfection.

4. As might be expected, the massive mortalities produced by chytridiomycosis severely compromise the variability and genetic structure of populations. Therefore, in order to maintain the greatest genetic diversity as possible, reintroduction programs should be based on genetic studies that allow optimal crossbreeding.

5. Different susceptibility of the amphibian species to chytridiomycosis causes that both the dynamics of transmission and the intensity of the infection vary according to the composition of the species of the community, being very difficult to predict the effect that would produce the arrival of the pathogen in a certain enclave.

6. Some species of amphibians behave as super-hosts, disproportionately increasing the infection of other species present in the community.

7. Monitoring amphibians with mark-recapture methods, although when they are developed over relatively short periods of time, allow for accurate demographic parameters, such as survival rate or recruitment rate, and population trends. However, this information must be contextualized with abiotic and biotic factors, since the

environmental conditions and chytridiomycosis can also influence population dynamics.

8. The most susceptible species to chytridiomycosis experience very pronounced and prolonged population declines that vary in intensity according to their different susceptibility to disease. However, at least in mountainous areas, climate change frequently determines population trends, and a combined analysis is necessary to accurately establish the incidence of the disease.

EPÍLOGO

LAS CUATRO RANAS

El saber y el medio saber

Estaban cuatro ranas sentadas sobre un grueso tronco de leña que flotaba a la orilla de un anchuroso río. Una ola furiosa arrastró al tronco hasta la mitad del río, donde la corriente lo condujo con el curso del agua. Alborozáronse las ranas por el encanto de su expedición y comenzaron a saltar sobre el tronco porque jamás se vieron navegar mar adentro. Pasado un momento de silencio la primera rana gritó:

- ¡Qué tronco más curioso y extraño! Mirad, compañeras, cómo viaja igual que los seres vivientes. Jamás he visto ni oído hablar de cosa tan parecida.

La segunda rana: - Este tronco no camina, se mueve amiga mía; y tampoco es extraño y curioso como te lo has imaginado. Las aguas del río que corren de por si hacia el mar conducen con ellas a este tronco que a su vez nos conduce con él.

La tercera rana: - No, por mi vida, compañeras, os equivocáis. Es una divagación la vuestra. Ni el río se mueve ni el tronco. Es nuestro pensamiento el que se mueve dentro de nosotros y él es quien nos conduce a creer en el moviemiento de los cuerpos inmóviles.

Discutieron largamente las tres ranas sobre qué era lo que se movía en realidad, llenando la quietud del río con sus gritos y su perturbador croar.

Como no llegaron a ningún acuerdo, pidieron la opinion de la cuarta rana. Esta, que hasta entonces no había dicho esta boca es mía, sino que las escuchaba con atención, habló de la siguiente manera:

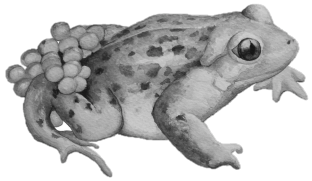
- Todas vosotras habéis tenido razón, compañeras, y ninguna se ha equivocado en sus razones. El movimiento está en el río tanto como en el tronco, como en nuestro pensamiento al mismo tiempo.

Este fallo conformó a las tres ranas en disputa, porque cada una quería tener la razón.

Cuéntase que lo que sucedió después del fallo de la cuarta rana fue cosa curiosa en el reino. Las tres ranas hicieron la paz entre ellas y en un conciliábulo ejecutivo resolvieron echar a la cuarta rana al río.

Y la arrojaron al agua.

Khalil Gibran



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